The World Health Organization (WHO) is a specialized agency of the United Nations and represents the culmination of efforts to establish a single intergovernmental health agency. As such, it inherits the functions of antecedent organizations such as the Office International d'Hygiène Publique, the Health Organization of the League of Nations, and the Health Division of UNRRA.

WHO had its origin in the proposal made at the United Nations Conference held in San Francisco in 1945 that a specialized agency be created to deal with all matters relating to health. In 1946, representatives of 61 governments met at the International Health Conference, New York, drafted and signed the WHO Constitution, and established an Interim Commission to serve until the Constitution could be ratified by 26 Member States of the United Nations. The Constitution came into force on 7 April 1948, the first World Health Assembly met in Geneva in June 1948, and on 1 September 1948 the permanent Organization was established.

The work of the Organization is carried out by three organs: the World Health Assembly, the supreme authority, to which all Member States send delegates; the Executive Board, the executive organ of the Health Assembly, consisting of 18 persons designated by as many Member States; and a Secretariat under the Director-General.

The scope of WHO's interests and activities exceeds that of any previous international health organization and includes programmes [illegible] malaria, tuber- [illegible] and [illegible] on, nursing, environmental sanitation, public-health administration, professional education and training, and health education of the public. In addition, WHO undertakes or participates in certain technical work of international significance, such as the compilation of an international pharmacopoeia, the setting-up of biological standards and of standards for insecticides and insecticide-spraying equipment, [illegible] exchange of scientific [illegible] collection and dissemination of epidemiological information, and statistical studies on morbidity and mortality.

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INFLUENZA

A Review of Current Research

INFLUENZA

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CONTENTS

INTRODUCTION

In fulfilling its constitutional role as the directing and co-ordinating authority on international health work, the World Health Organization has since its inception devoted considerable attention to the problems of influenza. Its Expert Committee on Influenza has recently been considering* how best to promote international collaboration in the control of this disease, while since 1947 the WHO influenza programme, details of which will be found in an article by Dr A. M.-M. Payne in this volume, has been developing rapidly. Under this project 54 laboratories in 42 countries have voluntarily co-operated in an international programme of research which could not be undertaken on this scale in any other way.

It must be recognized, however, that fundamental research and the development of new techniques, at least in their initial stages, are best carried out through private or institutional initiative, almost all significant scientific advances in the past have sprung from such efforts. The role of WHO is to facilitate this work by various means—for example, by collecting and distributing epidemiological information and laboratory findings on an international scale, by promoting the rapid exchange of scientific information among the various workers throughout the world, by providing for the training of specialized workers, and by supplying standard diagnostic reagents and other laboratory needs. All these activities are currently being undertaken by WHO

The production of this monograph falls within the second category, that of promoting the exchange of scientific information Many workers have not the time to read the vast literature on influenza, even if they have access to a large well-stocked library, which is not always the case By bringing together in one volume reviews of various aspects of this complex and fascinating subject, written, at special invitation, by some of the world's leading authorities who through their own work have contributed so greatly to our knowledge, it is hoped to bring to influenza workers the latest information, to show them where further detail can be found, and to stimulate those engaged in routine work to step out into the field of research

These articles have a practical value Those on the epidemiology of influenza by Dr C H Andrewes, and on influenza virus vaccines by Dr T Francis, Jr, will help public-health authorities to understand the

* World Hlth Org techn Rep Ser 1953, 64

problems of the correct use of vaccines. The paper on the causation and treatment of influenzal pneumonia by Professor J. Mulder and Professor C. H. Stuart-Harris will be of value to all clinicians. The article by Sir Macfarlane Burnet on the somatic and genetic aspects of the influenza virus, and that by Dr. P. von Magnus on its morphology, immunology, and kinetics of multiplication, will surely awaken a desire to undertake the fundamental research which is so essential to progress. The laboratory worker will acquire valuable technical information from the article by Professor P. Lépine, and both epidemiologists and virologists will find much to interest them in the paper on influenza antibodies in the population of the USA by Dr. M. R. Hilleman and his colleagues, as well as in the statistical article by Mr. Z. Deutschman. Finally, a classified bibliography has been provided to help workers to explore recent literature. It is not meant, however, to be complete in itself, and is complemented by the reference lists of the individual papers.

This monograph is not intended to replace the textbook: much that can readily be found in the standard works has been deliberately omitted. It is rather both complementary and supplementary to the textbook, covering the most important aspects of recent advances, and offering a range of speculation beyond the province of the orthodox textbook.

EPIDEMIOLOGY OF INFLUENZA

C. H ANDREWES, M.D, F.R.C.P., F.R S

In charge of the World Influenza Centre of the World Health Organization, and Deputy Director of the National Institute for Medical Research, London, England

The behaviour of influenza has [illegible] rence in both time and space th[illegible] many years Recent work seems [illegible] variable and plastic than are mos[illegible] and it may well be that this variability can be held largely accountable for its vagaries. The history of modern research on influenza dates from the discovery in 1933 of the susceptibility of ferrets to the virus. Since then we have been able to say whether or not in any "influenza" outbreak we are dealing [illegible] cular disease agent As to the [illegible] [illegible] [illegible] made, for convenience, that most [illegible] influenza of the last century has been due to viruses A or B, or to variants of them

History of Influenza Epidemics, 1850-1950

The earlier influenzas, of which [illegible] 1820 and 1850, will not be discus[sed] [illegible] into four periods :

(1) Up to 1889 influenza was at a low ebb and was becoming an almost extinct disease

(2) A period was ushered in by the pandemic of 1889-90; influenza then became, and has since remained, an important cause of mortality and morbidity in most temperate climates

(3) In 1918 influenza reached pandemic proportions and is believed to have killed 15-20 million people within a couple of years Its special character was a tendency towards bronchopneumonic complications fatal to previously healthy young adults. In the years following it gradually became less virulent.

(4) Since about 1933 influenza A has come to many countries in outbreaks every two or three years, with a tendency as time has passed for

in non-epidemic years—or they show it very rarely. Yet there is some respiratory infection which kills people every year and which was not widely prevalent before 1890. The writer [1] has discussed whether, between epidemics, influenza virus may not persist as a "basic virus" which cannot be recognized as influenza by current laboratory tests. Such a virus could perhaps be held accountable for the "influenza" deaths of "non-influenza" years. Otherwise we must believe either that the statistics are meaningless, or that influenza virus is present but is overlooked in some years, or that some quite different respiratory infection has enjoyed increased prevalence, alongside influenza, since 1890.

The 1918-19 pandemic and after

This, the most lethal pandemic known in history, will not be discussed except in relation to the general theme. According to Frost,[11] there had been a steady increase in the mortality from influenza and pneumonia in New York between 1914 and 1918. In view of recent experiences, it is very questionable whether this progressive rise can be ascribed to the activity of influenza A or of any other single virus. We do not, in fact, know at all what led to the catastrophic events of 1918-19. In the early months of 1918 the influenza had no unusual properties, but in June, in Britain, there was some indication that it was beginning to kill young adults. The really virulent form of the disease appeared at about the same time in the United States of America, around Boston, and in France, in Brest —i.e., at ports of embarkation and debarkation of American troops coming to Europe. In the ports of western Europe, too, there were people of many other races. In view of recent work on gene-recombination among influenza viruses it might be argued that a vicious new hybrid virus had been born in that very mixed culture. (Burnet & Lind,[7] it will be recalled, found evidence that, experimentally, the properties of two influenza viruses could be exchanged, producing a stable variant having mixed attributes.)

The rapid spread of the lethal kind of influenza all over the world is a familiar story. It is estimated that at least 15 million people died as a result. The island of St. Helena in mid-Atlantic is one of the very few places known to have definitely escaped. It seems likely that during the pandemic several different mutants may have arisen, having differing antigenic properties but all still sharing the ability to kill young adults. In such a way we can perhaps account for the fact that, in some English schools and other communities, later waves of influenza might or might not spare the victims of earlier waves.[12] A partial but incomplete protection by one strain against the effects of another could be invoked to explain the varying number of waves which hit different cities in the USA (Pearl [26]). We may picture a number of virus outbreaks moving around, being deflected from their courses when they met with foci of greater resistance.

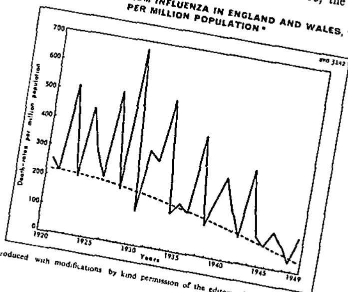
An interesting question of some practical importance is raised by the considerable postponement of the entry of pandemic influenza into Australia · this is claimed to have been due to the imposition of quarantine (cf. Burnet & Clark [9]) The quarantine was, however, far from complete, and experience with the influenza of these times tells us that many carriers and subclinical cases occur. It is quite likely, therefore, that Australia's immunity up to the beginning of 1919 can be attributed rather to the prevalence there of what we may call a routine outbreak of ordinary influenza in September-October 1918

After the pandemic years influenza returned gradually to its former habits of killing the old and weak rather than the young and vigorous. This reversion took at least three years in the USA and perhaps a decade in Britain We may suppose that the 1918-19 influenza, aggressive as it was, lacked some property enabling it to persist between epidemics, so that a less spectacular derivative, or perhaps the resurrected pre-pandemic strain, gradually replaced it.

Recent history

There has, perhaps, been a steady change in the epidemiology of influenza even since the pandemic, but it is convenient to treat "recent history" as being from 1933 when the influenza virus was first transmitted to ferrets The changing death-rate in England and Wales has been discussed by Martin [24] It seems that, especially since 1933, the mortality

FIG 2 DEATH-RATES FROM INFLUENZA IN ENGLAND AND WALES, 1920-1949, PER MILLION POPULATION *



* Reproduced with modifications by kind permission of the editors of the *British Medical Journal*

from influenza has been falling and the peaks have been tending to be smaller and more widely spaced. It is true that there was a big peak in 1951 in Britain and in much of Europe, but we cannot yet assess whether that was just a "flash in the pan". Fig. 2 reproduces a chart from Martin's paper, to show this tendency; it has been modified by inserting a line joining the "troughs". Such a line indicates deaths from "influenza" in the years when no influenza virus is recovered. Something has apparently been declining just as remarkably as the influenza which produces sharp outbreaks. As discussed earlier, this "something" could be a "basic influenza" not recognizable by laboratory tests, or some other disease which became important when influenza did in 1890 and is now declining pari passu with it. Martin thinks that the "decline in mortality is certainly not due wholly to the use of antibiotics, although it may have been accelerated by it".

Antigenic Studies of Influenza Viruses

In recent years it has been possible to isolate viruses from each epidemic and thus to discover much more than is possible by the mere study of vital statistics. Since 1940 we have known that there are two influenza viruses, A and B, having nothing in common antigenically, and therefore likely to behave epidemiologically as separate entities. It has been suggested that in the USA visitations of influenza B have tended to come every four or six years, and those of A every two or three. In some outbreaks, serological evidence of double infections with A and B has been rather frequent and it is difficult to be sure that epidemiologically the two agents act quite independently. There is no evidence as yet that influenza C (Taylor,[32] Francis, Quilligan & Minuse[10]) is of much epidemiological importance.

What seems very clear is that major influenza outbreaks can mostly be ascribed to influenza virus A. Great interest therefore attaches to the antigenic variations which have been occurring among the A viruses since 1933. Antigenically, the original (1933) A virus, WS, stands somewhat alone. It had been passed many times in ferrets and mice before antigenic comparisons with later strains were made, and it may have become modified during that time. In the few years following 1933 a few other strains antigenically similar to WS were recovered, but at that time the danger of picking up strains as laboratory contaminants was not appreciated, and we must beware of accepting unreservedly the view that all early isolations of A were of strains closely related to WS; it may well be, however, that 1933-5 was the end of a period when WS-like strains were prevalent.

During the decade 1936-46 the influenza strains isolated from all parts of the world were more or less closely related serologically to the PR8 strain recovered from Puerto Rico by Francis in 1934, and to the

Melbourne virus isolated by Burnet in Australia in 1935. Within this group there were minor variations such as those detected in southern England in 1936-7 (Stuart-Harris et al [30]) Also, in 1943, the Weiss strain was sufficiently different from PR8 for homologous (Weiss) vaccines to give particularly good results in a vaccination trial in the USA (Commission on Influenza [33]) Still, all the strains from this decade were fairly closely related.

In 1946 in Australia, a strain (Cam) was isolated, and proved to be somewhat removed antigenically from those previously prevalent. Its significance was not at first appreciated, but in 1947 similar viruses were recovered in numbers in North America and also from Europe (Sweden, the Netherlands, and Great Britain). In the USA, these strains were called A-prime (A′) strains, to indicate a major divergence from the classical A viruses; strain FM1 from the USA is regarded as a typical representative. Since 1947, strains related to FM1 have been recovered from A outbreaks in every continent, and seem, quite suddenly, to have completely replaced those related to PR8 It is true that a few workers have reported isolation of viruses closely related to PR8 From their first isolation, however, all these strains have been virulent for mice—a thing which did not happen in the era when PR8-like viruses were of widespread occurrence. Andrewes et al [2] have made clear the dangers of an accidental "pick-up" of a contaminating virus in the laboratory; and Isaacs & Andrewes [19] have adduced arguments for believing that such pick-ups may be responsible for all or most of the isolations of PR8-like viruses in recent years

There is a general belief among those studying the antigenic characters of viruses that a succession of antigenic changes is occurring. Viruses resembling WS have not appeared since 1935 or thereabouts, having been replaced by those related to PR8. Since 1947 these too have gone, and the A-primes hold the stage In the PR8 decade there were variations on the main antigenic theme, and nowadays variations are played from year to year on the theme of FM1. Isaacs, Gledhill & Andrewes [21] felt that, by refined serological methods, they could distinguish in most instances between the A-primes of 1947, of 1949, and of 1951. While almost all workers are agreed as to the existence of progressive antigenic changes among A viruses, there is considerable divergence of views as to detail, depending on the techniques used. Thus, British workers take the view just expressed—that there was a decade of prevalence of strains related to PR8 and that the A-primes which have held the stage since 1947 differ from each other in minor respects and can all be considered members of one group These opinions are based on studies with immune sera prepared in ferrets The ferret sera have shown up an epidemiologically important difference between two strains occurring in 1950-1—"Scandinavian" and "Liverpool" substrains, to be referred to later. Magill & Jotz, [22] like other American workers, have made antisera in rabbits,

which, unlike ferrets, are insusceptible to actual infection and have to be immunized intravenously or intraperitoneally rather than by *intranasal infection.* These workers concur in finding that PR8-like strains replaced the WS type about 1934, and that these in turn gave way to different *viruses in 1946-7. They encountered two strains,* one from 1936, one from 1940-1, with properties of somewhat intermediate nature. They, too, find changes in prevalent viruses from 1947 to 1948-9 and from then to 1950-1, but do not, as do Isaacs, Gledhill & Andrewes,[21] sharply differentiate between a major change from the PR8 era to the A-prime era, and *relatively minor changes occurring since.*

Hilleman,[14] using roosters to make antisera, has reached conclusions broadly similar to those of Magill. In studying a series of strains isolated in 1933, 1943, 1947, 1948, 1949, and 1950 he finds evidence of gradual loss of "earlier" antigens and an emergence of new ones.

Hirst [16] has used rabbit sera and by absorption tests with *large quantities of virus has studied individual antigens; he has distinguished between* [illegible]

[illegible]

another in 1937, and one in 1941-3; then there have been the A-primes since 1947. Unlike Magill, who studied few pre-1947 strains and many recent ones, Hirst's viruses were more regularly spaced in time, but he *used only one post-1947 antiserum in his studies. He suggests that*

"prior to 1940, a wide variety of strains were prevalent at one time, even in the same epidemic, while since 1940 the existence of a single variety of strain throughout the world has been the dominant characteristic".

In the writer's opinion, Hirst's tables do not very convincingly support *this conclusion.* Differences of view among workers are closely related to the use of different animal species for making antisera. Sampaio [28] has made comparisons between antisera made in susceptible species (ferret and hamster) and in *insusceptible ones (rabbit and rooster), and* has shown that the latter wholly fail to differentiate between the 1951 Liverpool and Scandinavian strains, which behaved quite differently epidemiologically It seems likely that such inconsistencies as exist would be resolved if all workers used the same techniques. Possibly the use of ferret or hamster sera, *combined with an absorption technique,* would give the maximum information.

One may perhaps sum up the antigenic changes which virus A seems to have undergone by an elaboration *of the metaphor used earlier.* Over a period of years, variations may be played upon one antigenic theme, *but after* some time the possibilities will be exhausted (the herd will be generally resistant to closely related variants), and the introduction of a new motif will be necessary to keep things alive. New variations again upon this will be possible.

Some writers think that the virus has unlimited capacity for variation, others that this is not infinite and that one day we shall inevitably be brought back, as in so many musical compositions, to the original theme. Francis [8] thinks such a circular course would afford "a splendid basis for the classical notions of the periodicity of influenza".

Studies of Epidemic Spread in Recent Years

In 1945-6 influenza B showed very widespread activity. It was active in the Pacific (Guam, Hawaii) about June 1945 and thence seemed to spread south to Australia, east to the Caribbean, to South and North America, and later to Europe (1945-6). This worldwide activity drew attention to the desirability of some concerted attempt to study the global epidemiology of influenza. After discussions at the Fourth International Congress on Microbiology held in Copenhagen in 1947, the World Influenza Centre (WIC) in London and its network of associated laboratories came into being as one of the activities of WHO. In the winter of 1948-9 came the first opportunity to test the value of the arrangements for observing the spread of an epidemic in correlation with antigenic studies of the viruses concerned (Chu, Andrewes & Gledhill [6]). News came first of an outbreak in Sardinia in September 1948. Magrassi [21] noted that this was apparently multicentric in origin, starting almost simultaneously in ten inhabited localities. Very soon after, influenza was reported in Sicily and Calabria (November 1948) and then along the length of Italy (December 1948 to January 1949) and in Switzerland, Austria, France, the Netherlands, western Germany, and northern Spain. About the New Year of 1949 virus was isolated in southern England, but the morbidity was less than in most of continental Europe and the evolution of the outbreak was slow. It spread also to Iceland and Denmark, but the force of the wave was spent. In Ireland and Sweden the existence of A infection was determined serologically, but there was no epidemic.

Two interesting things emerged from the study of this outbreak. Strains were sent to the WIC laboratory from Italy, Switzerland, France, the Netherlands, Britain, and Iceland. All were A-prime and were serologically quite homogeneous, differing slightly but recognizably from A-primes of previous years. It thus seems clear that a single type of virus was concerned in the outbreak which covered all western Europe. Belief in actual spread of a virus seems inescapable, although the spread may, of course, have occurred some time before, rather than immediately before, the appearance of a visible epidemic.

Magrassi [21] writes that "migration of an individual carrier of infection into new groups of population is insufficient to give rise to influenza" [a]

[a] "... non è sufficiente la migrazione dell'individuo portatore dell'infezione in nuovi gruppi di popolazione per diffondervi l'influenza."

It is noteworthy that it took some months for the September influen in Sardinia to involve the rest of Italy; but from Rome to Iceland tl passage was very swift. It is also remarkable that the 1948-9 influen did not apparently spread to eastern Europe. At any rate, influenza was not very active in Czechoslovakia or Hungary : as to Poland, we a less certain. Possibly a comparatively limited interference with freedo of travel hampers spread of the infection.

The other point of interest is that influenza first arose in Sardini Sicily, and Calabria—provinces in which there had been a small outbre in the early summer of 1948 Some of this infection was apparently du to influenza B, but the circumstances recall events of 1943 when, in Britai Canada, and the USA, an autumn epidemic was preceded by a sma outbreak in the same areas in the early summer. In Britain the sprir outbreak had been of influenza A following B. It seems possible th virus which fails to start an epidemic in the, for it, unfavourable seasc of early summer, may " go underground " and become seeded in a wid spread manner, to be activated by unknown factors and appear " mult centrically " in the autumn.

In the winter of 1949-50 there was virtually no influenza in Europ but in June 1950 the A-prime virus became locally active in Swede This virus was serologically a little different from that of 1949; and it wa with much interest that one learned of the beginning of influenza i Denmark in October 1950, and soon after in Sweden. Again a local summe outbreak had appeared, died down to vanishing point, and reappeare in the same area in the autumn (Isaacs & Andrewes [19]) Epidemic influenz was reported before long in Norway, Finland, Iceland, western German and the Low Countries, and about Christmas-time on the eastern coas of England and Scotland. Viruses from most of these places were obtaine and proved to be A-primes similar to that isolated in June 1950; the were called the Scandinavian subtype. A similar virus apparently becam active in southern Ireland about December 1950 and spread north (Meena & Clarke [25]). There did not occur, however, a regular spread over wester Europe from Scandinavia similar to the northward march of 1948-9 In Britain, a week or two after influenza had appeared in Aberdeen an Newcastle, very possibly from across the North Sea, a much more viciou type became active around Liverpool, killing many old and infirm peopl and causing considerable disquiet Virus from here was also an A-prim but, with ferret and hamster sera, was readily distinguishable from th Scandinavian strains. This Liverpool subtype became prevalent soo afterwards in Belfast and was recognized in other parts of England although there it did not show the same killing powers as in Liverpool It soon appeared that viruses sent in from influenza then present i France, Spain, Italy, Greece, Turkey, and Palestine were of the Liverpoo type. Further, these viruses were identical with some which had bee

received from epidemics that had occurred in Melbourne, Johannesburg, and Cape Town six months or so earlier. Viruses subsequently occurring in Canada, and at least one from the USA, were also Liverpool type. A tentative explanation of these findings is that the influenza in north-west Europe had a double origin—partly from the activation of a dormant virus in Scandinavia, partly from a strain coming up from the southern hemisphere. A line drawn from Dublin to northern England, and so to the Netherlands and Italy, leaves most of the isolations of Scandinavian virus to the north and east and of the Liverpool variety to the south and west.

Influenza in 1952-3

In December 1952 influenza A appeared almost simultaneously in several parts of the northern hemisphere—Japan, the central USA—and, at least by early January, in France and southern England. In France it appeared simultaneously in widely separated parts of the country. By the middle and end of January it was prevalent in southern Germany, Switzerland, and Iceland, and was also widespread in the USA and Canada. Denmark was having a double epidemic, caused mainly by A, although the role of B was far from negligible. Sweden had a good deal of influenza, laboratory tests revealed B only but A may have been present also. By the beginning of February influenza had reached the Netherlands, Finland, Yugoslavia, Italy, and Portugal. In Britain its attack was for long concentrated mainly in the south, especially in and around London, only rather slowly did it spread to northern England and Scotland, and even then its attack was less severe than in the south. This is of interest since, two years before, the north was hit severely while southern England escaped quite lightly. Up to mid-March Ireland was unaffected, having only a little influenza B. Whereas in 1948-9 influenza apparently began in Sardinia and in 1950-1 first news was definitely from Scandinavia, the 1952-3 outbreak seems to have started almost simultaneously over a considerable area of western Europe. Not only that, but the European outbreak came very little later than those in Japan and North America.

A viruses from all the countries mentioned above were examined, all were A-prime and all but a very few were like the 1951 Scandinavian subtype. They differed, however, considerably among themselves as regards their P-Q behaviour (see page 22). Table I shows the behaviour of strains examined in London in 1950-1 and 1952-3 respectively.

Nine strains resembled the Liverpool 1951 subtype. They were found as follows 2/2 from Paris, 1/3 from southern France (Toulon), 2/2 from Switzerland, 3/3 from Portugal, and 1/10 from Finland. No important differences were found between the Scandinavian subtype strains from Japan, the USA, and Britain. It would appear that for one reason or another the Liverpool subtype had a poorer ability to survive than the Scandinavian

TABLE I. A-PRIME VIRUSES EXAMINED IN LONDON

Date	Number of strains	S (Scandinavian)	L (Liverpool)	Intermediate
		all strains		
1950-1	96	46	47	3
1952-3	93	84	9	0
		United Kingdom strains		
1950-1	13	4	8	1
1952 3	52	52	0	0

The facts of this epidemic suggest that "hidden" Scandinavian virus persisting from the 1950-1 outbreak may have become activated simultaneously in widely separate centres—Japan, the mid-west of the USA, France, and Britain. Later occurrence in more distant areas such as Iceland, Finland, Yugoslavia, Italy, and Portugal may well have been due to epidemic spread. The 1952-3 outbreak was not preceded by any recognized warning sign in the affected areas in early summer, as had happened previously; it was also unusual in that there was no local prevalence in the autumn before the major epidemic.

The origins of influenza A epidemics

These studies have an important and obvious bearing on the vexed question of the origins of influenza epidemics. Some maintain that influenza never arises from within a country but always comes from abroad. It is so hard for any worker to find influenza virus in his own country between epidemics that this view is only natural. One might, on superficial examination, think it easy to imagine that the virus could flit from country to country whenever it found the inhabitants susceptible, till it came back to its starting point and set off another epidemic, two years or so later. It is, however, very difficult to find influenza anywhere for long periods. Even if one imagines an annual swing between northern and southern hemispheres, one has to remember that influenza A does not break out in Europe every winter.

The alternative view is that influenza, between outbreaks, can go "underground", possibly in chronic lung lesions in man, possibly in a form unrecognizable by conventional tests ("basic virus"). Shope[29] has in fact shown that the related virus of swine influenza can survive for months or years in pig lung-worms which pass stages of their life-cycle within earthworms. The virus cannot be revealed within these worms either directly or by any immunological or microscopical test, but only by infecting pigs orally and then "provoking" infection by various non-specific stimuli. If such is the state of affairs with swine influenza virus,

it is not difficult to believe in the existence of similarly "masked" human influenza virus. We have, of course, no reason for suspecting a helminth intermediate host or carrier for the human virus; nor do we know what non-specific stimuli could activate the human disease.

It appears that when influenza virus is introduced into a country one of two things may happen. It may be imported in its fully-fledged and active state, and set off an epidemic forthwith. This apparently happened when influenza was introduced into Fort Barrow in Alaska in 1935 (Pettit, Mudd & Pepper[27]); also when coolies from China brought the infection to Ocean Island in the Pacific in 1948 (Isaacs et al.[20]). More often, however, events are less dramatic: the virus introduced takes hold less readily and some time has to pass, something else has to happen, before the epidemic begins. When a vessel touched at Angmagssalik in Greenland in 1935, influenza was apparently introduced, but an epidemic did not start until two months later (Høygaard[17]). Many similar instances could be given. The idea that virus may be introduced and widely seeded, and only later produce an outbreak, is one which would help to explain many apparent anomalies in the spread of the disease.

Reasons for believing in the existence of hidden virus are several. There is our inability to find overt virus over long periods. There are the early summer "flurries" which on several occasions have preceded a widespread autumn epidemic caused by an antigenically similar strain. There are the multicentric origins of outbreaks, and the appearance of influenza simultaneously over large areas, the spread being almost too quick for any explanation based on human communications. On the other hand, the events of 1948-9 and many other years leave no doubt that country-to-country spread can occur. One concludes, therefore, that both theories of the origins of epidemics are probably true: that latent virus can probably become activated and start an outbreak, and that in favourable circumstances this may gather momentum and spread across national frontiers. Such happenings form one of the main objects of study of the WHO influenza programme, they will need to be watched for many years yet before the pattern becomes clear and we are able to foretell what will be the next move of the influenza virus.

Antigenic variation and epidemics

No doubt one of the factors determining the occurrence of an influenza epidemic is the immunity-level of the population. It is doubtless a rise in this which determines the end of an outbreak. There is good evidence that subclinical infection is very common and that the antibody level of the community as a whole rises during an outbreak.

It would seem that, when conditions are unfavourable for it, influenza disappears—either overseas or underground—and that it does not readily

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The origins of influenza A epidemics

These studies have an important and obvious bearing on the vexed question of the origins of influenza epidemics Some maintain that influenza never arises from within a country but always comes from abroad It is so hard for any worker to find influenza virus in his own country between epidemics that this view is only natural. One might, on superficial examination, think it easy to imagine that the virus could flit from country to country whenever it found the inhabitants susceptible, till it came back to its starting point and set off another epidemic, two years or so later. It is, however, very difficult to find influenza anywhere for long periods. Even if one imagines an annual swing between northern and southern hemispheres, one has to remember that influenza A does not break out in Europe every winter.

The alternative view is that influenza, between outbreaks, can go "underground", possibly in chronic lung lesions in man, possibly in a form unrecognizable by conventional tests ("basic virus"). Shope [29] has in fact shown that the related virus of swine influenza can survive for months or years in pig lung-worms which pass stages of their life-cycle within earthworms The virus cannot be revealed within these worms either directly or by any immunological or microscopical test, but only by infecting pigs orally and then "provoking" infection by various non-specific stimuli If such is the state of affairs with swine influenza virus,

it is not difficult to believe in the existence of similarly "masked" human influenza virus. We have, of course, no reason for suspecting a helminth intermediate host or carrier for the human virus, nor do we know what non-specific stimuli could activate the human disease

It appears that when influenza virus is introduced into a country one of two things may happen. It may be imported in its fully-fledged and active state, and set off an epidemic forthwith This apparently happened when influenza was introduced into Fort Barrow in Alaska in 1935 (Pettit, Mudd & Pepper [27]); also when coolies from China brought the infection to Ocean Island in the Pacific in 1948 (Isaacs et al.[20]). More often, however, events are less dramatic : the virus introduced takes hold less readily and some time has to pass, something else has to happen, before the epidemic begins When a vessel touched at Angmagssalik in Greenland in 1935, influenza was apparently introduced, but an epidemic did not start until two months later (Høygaard [17]) Many similar instances could be given. The idea that virus may be introduced and widely seeded, and only later produce an outbreak, is one which would help to explain many apparent anomalies in the spread of the disease

Reasons for believing in the existence of hidden virus are several There is our inability to find overt virus over long periods. There are the early summer "flurries" which on several occasions have preceded a widespread autumn epidemic caused by an antigenically similar strain. There are the multicentric origins of outbreaks, and the appearance of influenza simultaneously over large areas, the spread being almost too quick for any explanation based on human communications On the other hand, the events of 1948-9 and many other years leave no doubt that country-to-country spread can occur One concludes, therefore, that both theories of the origins of epidemics are probably true· that latent virus can probably become activated and start an outbreak, and that in favourable circumstances this may gather momentum and spread across national frontiers Such happenings form one of the main objects of study of the WHO influenza programme, they will need to be watched for many years yet before the pattern becomes clear and we are able to foretell what will be the next move of the influenza virus

Antigenic variation and epidemics

No doubt one of the factors determining the occurrence of an influenza epidemic is the immunity-level of the population It is doubtless a rise in this which determines the end of an outbreak There is good evidence that subclinical infection is very common and that the antibody level of the community as a whole rises during an outbreak

It would seem that, when conditions are unfavourable for it, influenza disappears—either overseas or underground—and that it does not readily

and automatically arise again, at least for several years In remote places, such as St. Helena and the Arctic, it probably dies out altogether. Perhaps it did so over much of the world between 1850 and 1890. When it does return, variants of somewhat novel antigenic make-up would seem to have a greater chance of survival than old strains, for which most people will have the appropriate antibody ready and waiting.

P-Q variation

Apart from its comparatively infrequent major antigenic changes, influenza A would seem to be capable of a less drastic type of variation. Van der Veen & Mulder [34] described P, Q, and R " phases " of A viruses. P viruses reacted to high titre in haemagglutination-inhibition tests with homologous sera, and to a much lower titre with heterologous ones; Q viruses reacted very poorly with all, even homologous, sera; the much less frequent R viruses were inhibited very well by all sera. It has been suggested (Isaacs & Andrewes [19]) that Q viruses, in a phase less readily suppressed by specific antibody, might be better able to survive in an immune population. Evidence was adduced that by laboratory manipulations a P-Q change in either direction might be induced. P viruses were made to become Q by passage in eggs in the presence of gradually increasing doses of homologous immune serum. Conceivably a Q virus is half-way to an undifferentiated " basic " virus. It was of interest in 1950-1 that all the Scandinavian strains isolated early in the outbreak were in the Q phase; only later did P-phase strains appear. The Liverpool virus, which seemed a more successful " spreader ", was probably all P-phase. Recently, Isaacs [18] has reported on two viruses isolated in the Persian Gulf in October 1952, 18 months after the 1950-1 outbreak was over and done with These consisted of a mixture of P- and Q-phase viruses related to Liverpool He raises the question of whether they may have been on the way " down " from P to Q; or whether, after a period " underground ", they were " coming up " and just acquiring P characteristics. A particularly interesting point is that the Q viruses concerned very closely resembled Q-variants of the Liverpool substrain, artificially produced in the laboratory in 1951 but not found occurring naturally at that time

Outbreaks of influenza B

So far only passing mention has been made of influenza B It tends to be more of an endemic disease than A, rarely initiating very large epidemics, and much more apt to cause merely local outbreaks in schools and other closed communities Some B type seems to occur almost every year, but it is hard to say how much its incidence varies from year to year.

In years when A is not about, small B outbreaks are likely to be better studied and their causative virus determined. When virus A is attacking in strength, a similar number of small B outbreaks might well be overlooked. Influenza B epidemics seem rarely to spread across frontiers; a possible exception is afforded by the worldwide activity of the disease in 1945-6.[8]

Virus B seems to be a better antigen than A. to this has been attributed the apparent wider spacing of its more extensive visitations. Also, vaccines made with it are possibly more effective than A vaccines, even if not made with the current strain of virus.

There are some differences of opinion as to the importance of antigenic variations among B viruses. All workers agree that the original (Lee) strain of Francis stands antigenically as apart from other strains as the original WS does from subsequent A viruses. Some American workers (Tamm et al.;[31] Hilleman et al.[15]) suggest that there have subsequently been antigenic changes in B viruses somewhat similar to those shown by A. On the other hand, Bozzo,[3] Brans,[4] and Hennessen[13] have found that almost all recent B viruses are much alike, even though isolated from different countries in different years. There is at present no suggestion of simultaneous worldwide changes in virus B such as occur with A, and this agrees with the notion that the activities of virus B are more apt to be parochial than international.

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INFLUENZA ANTIBODIES IN THE POPULATION OF THE USA *

An Epidemiological Investigation

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Early in the study of viral influenza it was noted that the immunology of this disease conformed to the basic principles established for other infectious agents Thus, infection is followed by specific antibody production and there is a rough but not absolute correlation between the amount of specific antibody in the circulating blood and the susceptibility or resistance of the host (Francis et al, [14] Rickard et al, [40] Salk et al [41]). Moreover, the kind and amount of specific antibody may be used as an index of previous infection with the virus, and information regarding the past occurrence of these agents may be obtained by determining the antibody level against the various types of virus in representative samples of the population

In the early investigations of this sort, the antibody levels were usually measured by the serum-neutralization test (Francis et al, [14] Horsfall et al, [22] Rickard et al [40]) This method was reliable, but costly in time and material, and has largely given way to the simpler haemagglutination-inhibition test of Hirst [25] Unfortunately, the value of the latter technique has been qualified because of the presence in animal and human sera of heat-stable mucoprotein substances (Hirst, [26] McCrea [33]) which inhibit haemagglutination by the influenza A and B viruses This inhibition is non-specific, and inhibitors are often present in such quantities that they

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prevent agglutination at higher dilutions than do the specific antibodies. This results in false positive antibody readings.

It was found that the inhibitory activity of this non-specific factor in serum is destroyed by treatment with the filtrate of cultures of *Vibrio cholerae* rich in a substance called receptor-destroying enzyme (RDE) (Anderson[2]). Moreover, the recent studies by Mulder and associates[36] and by Tyrrell & Horsfall,[47] as well as unpublished investigations in this laboratory, have shown that this cholera-filtrate treatment will remove the non-specific inhibitor of influenza A and B viruses from serum without destroying significant amounts of antibody. Hence, it is now possible to measure accurately the antibody in human serum against these two viruses by the haemagglutination-inhibition method. Recent investigations in this laboratory (Hilleman & Werner[24]) showed that the 1233 strain of influenza C virus was not inhibited by the non-specific factors in human serum. For this reason it is not necessary to treat the sera with cholera filtrate in order to measure the antibody against this agent.

This article describes the findings of a study made on human serum to determine the relation between the distribution of specific influenza antibody in the population of the United States of America and the prevalence of the various types of influenza in recent years. The population was sampled in two ways. The first method employed sera collected during 1951 from persons of various ages, while the second employed sera collected from representative groups of adults during each calendar year from 1943 to 1951, inclusive. Although the samples were small, the findings obtained by each of these methods were in good agreement and, moreover, were consistent with those of virus isolation studies made during the same period.

Materials and Methods

Serum

The sera tested in this study were selected as representative of the population of the USA with respect to influenza antibody. Most of the specimens had been obtained for other purposes and had been stored for years. The sampling of the population was therefore by no means random, but, within the limits of the availability of serum, the selection could be considered reasonably representative.

All sera were obtained from military personnel or their dependants free from active respiratory disease at the time of collection. Specimens used in the tests for influenza A and B were from groups of persons different from those tested for influenza C. The children's sera[a] were from patients hospitalized for non-respiratory causes in the summer and autumn months

[a] We are indebted to Colonel O. C. Bruton, M.C., Chief, Pediatrics Section, Walter Reed Army Hospital, Washington, D.C., USA, for these sera.

of 1951. The adult sera dated prior to 1951 were from persons ill with non-respiratory disease during the summer and autumn months of the indicated year. The adult sera for 1951 were from soldiers in good health; those tested against influenza A and B were collected in August, and those used in the tests for influenza C antibody were drawn in February. All specimens were stored at −20°C in this laboratory from the time of their collection

Cholera-filtrate preparation

V. cholerae strain 4-Z was used for the preparation of cholera filtrate. This agent was obtained from Dr B. Briody, who had received it from Sir Macfarlane Burnet. The filtrate was prepared by growing the organism for 24 hours at 35°-37°C in fresh beef-heart infusion broth containing 1% neopeptone and 0 5% sodium chloride. The pH of the medium was adjusted to 7 6 and the material sterilized in the Arnold sterilizer This represented a modification of the original method of preparation described by Burnet & Stone [5] The Seitz filtrate was adjusted to a pH of 7 0 and usually had an RDE titre of 1/256 when tested with egg-line FW-1-50 virus by the assay method of Burnet & Stone [5] It was highly effective in removing the non-specific inhibitor from serum but did not destroy any significant amount of specific antibody

Cholera-filtrate treatment of serum

All the sera to be tested against influenza A and B viruses were treated with cholera filtrate to remove non-specific inhibitor by the method of Mulder, de Nooijer & Brans.[38] In this procedure, each volume of serum was incubated for 14-15 hours at 37°C with four volumes of the filtrate Residual filtrate activity was removed by heating the mixture at 56°C for 50 minutes Under the test conditions employed, influenza C virus (strain 1233) was found to be unaffected by non-specific inhibitor in human serum and, hence, the sera tested with this virus were not given cholera-filtrate treatment

Viral antigens

Antigens were prepared from strains of virus selected as representative of the various known types and subgroups of the agents of human influenza (van der Veen & Mulder,[50] Burnet,[4] Isaacs,[23] Hilleman;[19, 20] WHO[61]). These were as shown in table I

These viruses have been discussed previously (Hilleman et al [19, 22]) and have been used as prototypes for earlier strain-analysis studies (Hilleman [19]) The antigens were prepared by methods already described (Hilleman et al [21]) and were preserved in the dried state until used

TABLE I. STRAINS OF VIRUS USED FOR PREPARING VIRAL ANTIGENS

Virus type	Subgroup name	Test viruses employed	
		strain	year isolated
A	WS	WS	1933
A	PR8	PR8	1934
A	A-prime	FM1	1947
A	A prime (contemporary)	FW-1 50	1950
B	Lee	Lee	1940
B	Warner (synonym Bon)	IB1	1950
C	1233	1233	1947

Test procedure

The haemagglutination-inhibition titrations conformed to the technique of the Standard Reference Test in Influenza Diagnostic Studies (Committee on Standard Serological Procedures [8]) Type " O " human red blood-cells were employed in all tests. With influenza A and B viruses, the tests were read after the sera had stood for 55-60 minutes at room temperature. In the tests with influenza C, the sera were incubated in the refrigerator at 4°C for 60-65 minutes. Care must be taken in the reading of tests with [illegible] the bottom of the tube Unless the tests are carefully observed, they may be interpreted erroneously as negative agglutination.

[illegible] of the individual sera in the group. Differences in antibody level were considered significant if they were more than twice their standard error.

Findings

As might be expected, the antibody levels of the individual sera against any particular virus showed a considerable variation, and it was not until the mean levels of the groups were calculated and compared that the epidemiological pattern became clear. In the body of the report only the mean antibody levels for the various groups will be considered. However, illustrative examples of the amount of individual variation found are given in Annexes 1 and 2 (see pages 38 and 39). The mean antibody levels against the influenza A, B, and C viruses in sera collected in 1951 from persons of

TABLE II. MEAN ANTIBODY TITRES * OF SERA OBTAINED IN 1951 FROM PERSONS OF VARIOUS AGES

Age (years)	Number of sera	A		A prime		B		C	
		WS	PR8	FM1	FW 1 50	Lee	IB1	number of sera	1233
		geometric mean of titres							
< 1/2	7	2	13	1	6	9	2	0	—
1 2	16	0	2	2	9	2	2	6	4
3 5	31	2	7	15	41	7	2	27	63
6-8	30	2	7	44	64	6	3	24	80
9 11	25	2	10	68	52	10	8	10	121
adult	74	32	118	43	46	56	44	33	204

* Titres expressed as denominator of serum dilution

various ages are summarized in table II. The results of similar tests with the sera collected in the years 1943-51 from adults are given in table III These data are presented graphically and discussed in the subsequent sections of the paper.

TABLE III. MEAN ANTIBODY TITRES * OF GROUPS OF ADULTS, FREE FROM ACTIVE RESPIRATORY DISEASE, OBTAINED EACH YEAR · 1943-51

Year	Number of sera	A		A prime		B		C	
		WS	PR8	FM1	FW 1 50	Lee	IB1	number of sera	1233
		geometric mean of titres							
1943	35	9	18	3	5	10	7	32	253
1944	37	21	78	8	8	9	11	33	182
1945	33	14	65	13	8	24	20	33	123
1946	39	21	96	14	8	71	62	33	191
1947	32	28	91	18	20	65	43	33	159
1948	33	35	96	25	21	47	41	33	162
1949	35	23	83	19	21	69	39	33	219
1950	34	28	80	18	30	38	35	33	200
1951	75	32	118	43	46	56	44	33	204

* Titres expressed as denominator of serum dilution

Influenza A

Age-specific antibody levels. The mean antibody levels against the various subgroups of influenza A of those persons bled in 1951 are shown

Discussion. The findings described above are consistent with, and tend to confirm, our knowledge of the prevalence of the different types of A virus among the population of the USA. Thus, PR8 virus was isolated in 1934 (Francis[11]) and is representative of the A-type viruses commonly recovered over the ensuing ten years (Hilleman;[19] Magill & Jotz;[35] van der Veen & Mulder[49]). The latest epidemic attributed to this type virus occurred in the winter of 1943-4 (Collins & Lehmann;[7] Commission on Acute Respiratory Diseases;[48] Francis[13]) and the strain has seldom been recovered since that date (Lépine et al.;[32] Nagler et al;[37] van Rooyen et al.[39]). This distribution in time has resulted in an adult population with considerable past exposure to PR8 and a childhood population with little or no evidence of contact with this virus. Consequently, we find high antibody levels in adults and almost no antibody in children born since 1944. It is noted also that the latest significant augmentation of PR8 antibody in adults occurred after the epidemic in 1943-4. There are several possible explanations for the steady high PR8 antibody level maintained in adults since 1944 despite the apparent infrequency of the virus in the community. Perhaps homologous influenza antibody levels persist in persons for long periods of time. However, a more likely explanation would seem to be one involving an anamnestic response to antigenic components which are common to PR8 and A-prime viruses but which represent only a minor portion of the mosaic of the latter.

The A-prime viruses, on the other hand, have been predominant at least since 1946 (Anderson;[1] Chu et al.;[6] Hilleman,[19] Isaacs et al.;[30] Magill & Jotz;[35] van der Veen & Mulder[50]) but were not prevalent before that time. The FM1 strain was isolated in 1947 (Rasmussen et al[38]), while FW-1-50 was recovered in 1950 (Hilleman et al[23]) and represents the contemporary A-prime virus Epidemics of influenza ascribed to A-prime strains have occurred in the USA in the late winters of 1947 (Collins & Lehmann;[7] Francis et al;[17] Rasmussen et al;[38] Sartwell & Long,[42] Sigel et al[43]), 1950 (Collins et al;[7] United States, Influenza Information Center[49]), and 1951 (Collins et al.,[7] Davis[9]) Such a known distribution of A-prime viruses can be readily correlated with the data illustrated in fig 1, which indicate that children over six years of age and adults have similar antibody levels against these agents

The occurrence of the A-prime epidemics in 1947, 1950, and 1951 is reflected in the levels of the adult sera This is particularly true of the antibody against FW-1-50 It may be noted in fig. 2 that the antibody rise against FM1 began as early as 1945, and this may indicate the presence of this type virus at least one year before the strain was isolated

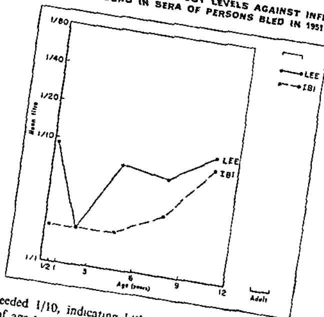
The WS strain was isolated in England in 1933 (Smith et al[44]) but has not been known to be prevalent since that time Because this was the first strain of human influenza virus to be isolated, nothing is known of when it first appeared or how long it had been present The almost complete

absence of antibody against this strain in children and the comparatively low levels in adults may be ascribed to the long interval that has elapsed since this virus has been known to be prevalent.

Influenza B

Age-specific antibody levels. The antibody levels against the Lee and IB1 types of influenza B of persons bled in 1951 are shown, by age, in fig 3. The mean titre of the children's sera against either of these viruses

FIG. 3 AGE-SPECIFIC MEAN ANTIBODY LEVELS AGAINST INFLUENZA B VIRUSES FOUND IN SERA OF PERSONS BLED IN 1951



never exceeded 1/10, indicating little exposure of the population under 12 years of age to these strains. In adults the antibody reached titres of 1/44 and 1/56.

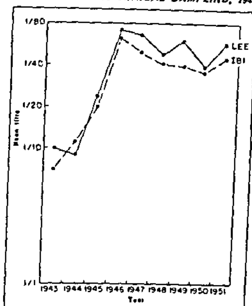
Annual sampling of adult sera, 1943-51 The yearly fluctuation in the antibody level of adults against influenza B is illustrated in fig 4 These sera showed low levels in 1943 and 1944, and significant increases in 1945 and 1946, thereafter, the mean titres were maintained at levels between 1/35 and 1/65

Discussion The Lee virus was isolated in 1940 (Francis[18]) and is not known to have been prevalent since that time, whereas the IB1 virus (Tamm et al[45]) was isolated in 1950 and is representative of the

subgroup of B viruses which have been isolated since 1943 (Brans;[3] Burnet;[4] Hilleman et al.;[19, 20, 22] Magill et al.;[35] Tamm et al.[45])

The serological patterns for B viruses, summarized in fig. 3 and 4, appear to be less closely related to the known epidemic prevalence of influenza B than are the periods and incidences of influenza A and A-prime, discussed earlier. The significant rise in B antibodies in 1946 and the subsequent maintenance of a high level in adults correlates with the 1945-6 epidemic (Collins & Lehmann;[7] Francis et al.;[16] Hirst et al.;[27] Kalter & Chapman[31]) and the intermittent prevalence of the virus since that time.

FIG. 4. MEAN ANTIBODY LEVELS OF ADULTS AGAINST *INFLUENZA B* VIRUSES FOUND BY ANNUAL SAMPLING, 1943-51



However, one wonders why the level remained in the neighbourhood of 1/10 during 1943 and 1944, only a few years after the 1940 epidemic of influenza B (Collins & Lehmann;[7] Eaton & Beck,[10] Francis;[12] Magill[34]), and why the antibody level increased significantly in 1945, one year before the 1945-6 epidemic. Moreover, the B antibody levels are relatively low in children of from 9 to 12 years of age who lived through the 1945-6 period. Certain observations may be considered in attempting to understand these aberrancies. These are concerned with the rather sporadic distribution of the disease, even during periods of prevalence of B virus and the relatively long periods between such episodes

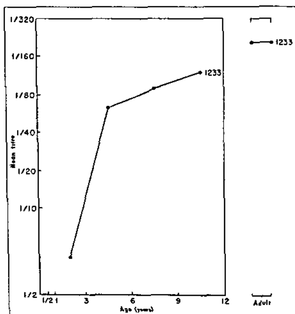
It is of interest that the antibody levels in human sera summarized in fig. 3 and 4 are similar for both strains However, the two agents can

be distinguished antigenically without difficulty in tests with animal sera (Hilleman et al ;[19, 22] Magill & Jotz,[35] Tamm et al[45]). The findings of this investigation seem to indicate that in human serum the tests measured the same antibody in spite of the antigenic differences demonstrable by other methods.

Influenza C

Age-specific antibody levels. The antibody levels against influenza C are shown by age in fig. 5 No sera were available from children of less

FIG. 5 AGE-SPECIFIC MEAN ANTIBODY LEVELS AGAINST INFLUENZA C VIRUS FOUND IN SERA OF PERSONS BLED IN 1951

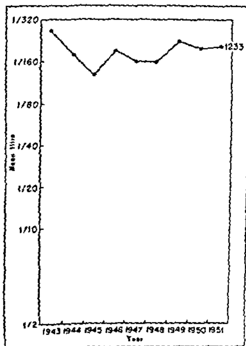


than six months of age at the time when the investigation referred to in fig. 5 was carried out Subsequent tests performed on the sera of three infants aged between six weeks and three months gave titres of 1/20, 1/40, and 1/320, this antibody was probably of maternal origin The antibody was at insignificant levels at from one to two years of age and then rose sharply to high levels at from three to five years This high level was maintained throughout all the older age-groups

Annual sampling of adult sera, 1943-51. The annual sampling of the adult sera shown in fig. 6 indicates a consistently high level of antibody throughout the nine years.

Discussion. Strain 1233, recovered from a case of respiratory disease in 1947 (Taylor[46]) was the first influenza C virus to be isolated. Its relation to clinical influenza was not clearly established until 1950 (Francis et al. [15]). Relatively few cases of this type of influenza have been reported to date, and few outbreaks have been described (Francis et al ;[15] Gerber et al.[18])

FIG. 6. MEAN ANTIBODY LEVELS OF ADULTS AGAINST INFLUENZA C VIRUS FOUND BY ANNUAL SAMPLING, 1943-51



In spite of this, the antibody level for influenza C in sera of children over three years of age is high and indicates that infection occurs at an early age. Furthermore, the presence of large amounts of antibody in sera collected from adults in 1943 indicates that the virus was widespread in the population before that time. These findings are in agreement with those reported by Francis et al [15] and support the concept that infection with this agent has been widespread in the population of this country for many years.

General Discussion

These studies utilized the diagnostic haemagglutination-inhibition method for the survey of influenzal antibody in the population The results of the tests with the influenza A and B viruses would not have been valid had not the sera been treated to remove non-specific inhibitor Unpublished studies from this laboratory have shown that the PR8 virus employed in the tests was especially affected by the non-specific substance Thus, it was observed that untreated sera from 16 children of from one to two years of age had a mean titre value of 1/160 with PR8; these same sera failed to give significant inhibition of haemagglutination after cholera-filtrate treatment. The WS, FM1, Lee, and IB1 strains were also markedly affected by the non-specific substance, but to a somewhat less extent, the FW-1-50 agent was relatively unaffected, and the 1233 strain of influenza C virus was not influenced by the inhibitor

In this study, the measurement of influenzal antibody in population groups was applied to the retrospective determination of influenza virus occurrence It is not unreasonable to expect that the same approach may be useful in predicting the susceptibility of a population to a particular virus This method might have been utilized at the time the first A-prime virus, Cam, was isolated in Australia in 1946 (Anderson[1]). Cam virus is essentially identical, antigenically, with the A-prime FM1 virus recovered in the USA the following year As shown in table III, the mean titre in adults for the FM1 virus in 1946 was only 1/14 This level was considerably below that for the A strains occurring previously, and studies of this type would have suggested the susceptibility of the population to this kind of virus Findings of this kind, if confirmed by a larger sampling of a representative group of persons, together with the results of the strain analysis studies, would suggest a need to include a newly isolated virus in the vaccine before the occurrence of an epidemic.

While the measurement of influenzal antibodies may provide a valuable yard-stick for estimating susceptibility of a given population to a given virus, it should be noted that the relationship between antibody titre and immunity is not always consistent Thus, it has been shown in studies of naturally and experimentally induced influenza that the disease sometimes occurs in persons with high antibody levels, and that persons with low titres may escape infection (Francis et al ,[14] Rickard et al ,[40] Salk et al[41]) It would appear, then, that other factors in addition to circulating antibody may be of importance in the immunology of this disease

ANNEX 1. ANTIBODY TITRES* AGAINST INFLUENZA VIRUSES IN SERA COLLECTED IN 1951 FROM CHILDREN BETWEEN THREE AND FIVE YEARS OF AGE

Serum no	Type A		Type A-prime		Type B		Serum no	Type C (1233)
	WS	PR8	FM1	FW-1-50	Lee	IB1		
38	0	10	0	10	0	0	6	10
80	10	20	80	160	10	10	10	320
122	0	10	40	40	0	0	14	320
123	20	40	160	160	40	0	16	40
126	0	0	0	0	0	0	23	320
141	20	80	160	160	20	0	25	320
147	—	—	—	20	10	0	26	320
155	0	0	0	10	0	0	31	80
167	0	0	0	10	0	0	32	10
171	0	10	10	20	20	0	33	160
178	0	0	80	160	0	0	34	320
179	0	0	320	640	10	0	36	0
180	0	10	320	640	20	0	37	20
185	0	0	0	0	0	0	38	40
186	0	40	0	40	40	0	39	80
192	0	10	160	320	0	0	41	40
194	20	40	640	320	40	10	44	20
195	0	0	160	80	10	40	53	160
196	0	20	320	320	20	20	55	320
197	0	20	0	20	20	0	62	160
198	0	0	0	10	20	0	63	80
199	0	20	160	160	20	0	66	20
210	0	0	80	80	0	0	72	10
215	0	0	0	20	20	0	77	10
216	0	0	0	20	10	0	78	160
219	20	40	0	20	40	10	81	10
220	10	20	40	40	20	10	86	640
222	0	20	40	40	0	0		
223	10	20	0	0	20	0		
232	20	40	320	320	20	0		
249	0	20	0	20	20	0		

* Titres expressed as denominator of serum dilution

ANNEX 2. ANTIBODY TITRES* AGAINST INFLUENZA VIRUSES IN SERA COLLECTED FROM ADULTS IN 1950

Serum no	Type A		Type A prime		Type B		Serum no	Type C (1233)
	WS	PR8	FM1	FW 1 50	Lee	IB1		
253	0	0	0	20	20	40	22	40
254	80	160	40	40	80	80	23	40
351	160	320	80	80	20	20	24	160
352	80	320	40	40	160	80	49	320
355	40	160	20	20	320	160	50	320
356	40	160	40	10	20	20	51	320
357	10	40	20	20	40	40	76	160
358	160	160	80	20	160	160	77	80
359	40	80	10	40	40	80	78	40
360	40	40	10	10	20	40	103	640
361	40	80	20	20	0	20	104	160
362	20	160	320	80	80	160	105	320
363	40	160	80	20	20	40	130	320
364	20	20	0	20	80	80	131	640
365	20	80	80	80	20	20	132	640
366	10	20	20	20	40	10	157	640
367	20	40	40	20	80	40	158	80
368	40	80	40	40	40	40	159	320
369	160	160	40	20	160	80	184	320
370	160	320	20	160	80	80	185	320
371	10	20	10	10	40	40	196	80
372	0	0	0	20	0	0	211	640
373	160	320	40	20	320	320	212	640
374	160	160	80	80	80	80	213	640
375	20	40	20	20	320	40	238	160
376	0	0	0	40	10	0	239	320
377	20	320	160	160	80	80	240	320
378	20	80	80	40	20	20	265	20
379	0	0	0	20	0	0	266	40
381	160	80	20	80	80	80	267	40
382	10	80	0	0	10	10	292	320
383	160	320	40	80	160	80	293	160
384	20	40	0	20	40	40	294	640
385	160	320	80	80	20	20		

* Titres expressed as denominator of serum dilution

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TREND OF INFLUENZA MORTALITY DURING THE PERIOD 1920-51

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During the thirty-two years following the great influenza pandemic of 1918-19, some fifteen epidemic waves of varying degrees of importance were recorded in the Northern Hemisphere. During this period, important developments affected the mortality ascribed to the disease The trends of mortality directly or indirectly attributed to influenza were affected by the wider use, in the treatment of the disease, of sulfonamides and, later, antibiotics

The gradual adoption of the identification procedures for the virus since it was first isolated in 1933 has added new information on the spread and incidence of the predominant types of the disease This new knowledge has already helped to clarify some aspects of the epidemiology of the disease hitherto designated "influenza".

During this important period in the history of influenza, knowledge of the amount of illness caused by the influenza virus has not materially improved, and few contributions have been made towards improving the reporting of influenza morbidity and the evaluation of mortality directly or indirectly connected with influenza epidemics

It is well known that, in countries where the disease is notifiable, the cases reported to health departments do not constitute any true index of morbidity and can hardly provide a measure of the relative importance of the successive epidemics. The reporting of influenza is both incomplete and irregular, although more incomplete in rural areas than in towns It is especially defective between two epidemics, but, as a rule, improves at the beginning of an outbreak and during the early part of the epidemic. In spite of their incompleteness, figures of influenza cases notified, if reported promptly by weekly or shorter periods, may serve as an index of the "explosiveness" and of the speed with which an epidemic breaks out and spreads, and are especially useful in its early stages They are, however, not suitable for the evaluation of the morbidity due to an epidemic.

Household surveys immediately following an epidemic—such as are carried out in the USA—yield valuable information on age-incidence, complications, and case-fatality not obtainable from any ordinary notification system Such occasional surveys do not, however, provide adequate data for estimating the total morbidity involved in an epidemic

Data on morbidity among selected population-groups are obtainable, in certain countries, from insurance records. Those collected by the Sickness Insurance Funds in pre-war Germany, for instance, provided a useful source of information on the progress of an epidemic in various industrial centres and on the total morbidity ascribed to influenza among the insured.

In the absence of other data, excess mortality from various causes is the main source of information on the importance of an influenza epidemic. Death figures from influenza and pneumonia in excess of the normal seasonal expectancy are commonly utilized as an index of the size of an epidemic.

Weekly death-rates in excess of seasonal expectancy were used by Collins and Collins & Lehmann [1, 2] in the study of influenza epidemics in the USA. An evaluation of mortality in influenza years in England and Wales by Martin [5] was based on excess deaths recorded in the quarter containing an influenza outbreak.

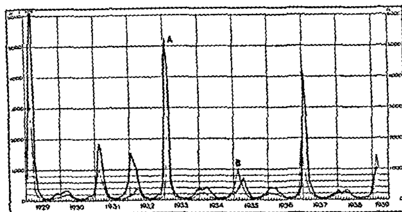
Most influenza epidemics, during the past thirty years, have been accompanied by a significant excess of deaths ascribed to causes other than influenza and pneumonia. During the period of the year corresponding to the influenza epidemic, excess mortality can be observed in most countries, not only from respiratory diseases, but also from heart diseases and other or ill-defined causes, particularly among elderly people.

The close connexion between mortality ascribed to influenza and the higher seasonal peaks of general mortality is well illustrated by curves relating to towns in England and Wales and in Germany in pre-war years (see fig. 1 and 2).

Deaths directly attributed to influenza represent only a fraction of the mortality involved in an influenza epidemic. The comparability of such influenza mortality-rates over a longer period of time is affected by several factors, as the data are influenced by changes in the method of certifying deaths, in reporting procedures, and—in some cases—in the rules applied in the selection of the cause of death, when several are mentioned on the death certificate. Furthermore, the differences in reporting procedures, and in the completeness of data in various countries, do not allow of international comparison of influenza mortality-levels. A varying proportion of deaths, according to the country, is attributed to pneumonia or bronchopneumonia, even when these are mere complications of influenza. In spite of the above-mentioned limitations, the annual influenza mortality-rates in various countries (see table I) show some striking similarities in trends over the period 1920-1951

Over the period 1920-51, high influenza mortality-rates in most countries of the Northern Hemisphere were reported during six to twelve out of the thirty-two years. Relatively high levels of mortality were recorded in the majority of European countries during the epidemic years : 1922,

FIG. 1. GENERAL AND INFLUENZA MORTALITY IN THE GREAT TOWNS* OF ENGLAND AND WALES AND IN THE GERMAN TOWNS† WITH A POPULATION OF MORE THAN 100,000: INFLUENZA MORTALITY



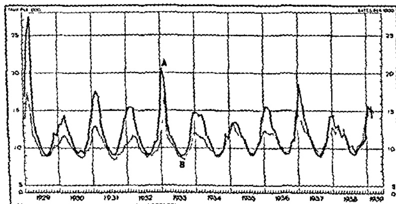
A = English towns B = German towns

* The number of towns has increased gradually from 105 in 1921 to 126 in 1939, and the total population from 19 to 21 million.

† The number of towns has increased gradually from 48 in 1921 to 57 in 1939, and the total population from 16.5 to 21.5 million

FIG. 2. GENERAL AND INFLUENZA MORTALITY IN THE GREAT TOWNS* OF ENGLAND AND WALES AND IN THE GERMAN TOWNS† WITH A POPULATION OF MORE THAN 100,000: GENERAL MORTALITY

(Rates per 1,000 Population, by Four-Week Periods, on an Annual Basis)



A = English towns B = German towns

* The number of towns has increased gradually from 105 in 1921 to 126 in 1939, and the total population from 19 to 21 million

† The number of towns has increased gradually from 48 in 1921 to 57 in 1939, and the total population from 16.5 to 21.5 million

TABLE I. ANNUAL INFLUENZA MORTALITY-RATES PER 100,000 POPULATION, 1920-51

Country	1920	1921	1922	1923	1924	1925	1926	1927	1928	1929	1930
Africa											
Union of South Africa [a]	.	82.8	50.6	25.3	30.7	24.4	39.0	24.4	26.9	19.4	23.8
America											
Canada [b]											
USA [c, h]											
Chile											
Uruguay											
Asia											
Japan	19.4–	18.2	22.1	11.2	11.0	18.1	7.6	13.2	15.5	13.4	8.1
Australasia											
Australia	8.4	12.0	6.4	21.3	10.0	5.9	12.5	6.9	12.7	15.9	4.3
New Zealand [d]	40.2	8.6	5.3	31.6	6.9	6.5	21.3	9.5	17.4	21.1	9.2
Europe											
Belgium											
Czechoslovakia											
Denmark [e]											
Finland											
France											
Germany [f]											
Greece											
Hungary											
Iceland											
Ireland											
Italy											
Netherlands											
Norway											
Spain											
Switzerland											
United Kingdom											
England and Wales	29.2	23.7	56.3	22.0	49.0	32.7	22.9	56.7	19.6	73.4	12.6
Northern Ireland [g]	44.5	25.1	70.1	33.0	81.6	55.8	33.4	59.6	31.0	107–	20.6
Scotland [i]	6.0	27.2	75.7	10.9	51.2	25.6	29.2	41.7	19.8	71.1	14.4

Country	1931	1932	1933	1934	1935	1936	1937	1938	1939	1940	1941
Africa											
Union of South Africa [a]	17.4	45.6	17.3	15.2	60.7	17.8	25.1	15.7	13.4	16.3	12.4
America											
Canada [b]	31.0	40.4	37.8	18.7	31.3	28.5	47.7	21.2	35.2	24.5	21.0
USA [c, h]	26.5	30.6	26.4	17.3	22.2	26.4	29.5	12.7	16.4	15.3	15.8
Chile	120.4	134.0	245.5	173.6	185.6	161.8	127.3	156.6	109.2	108.1	85.3
Uruguay					12.5	3.6	7.1	8.5	3.4	4.7	2.5
Asia											
Japan	24.0	8.1	7.1	14.8	4.4	12.4	4.2	10.7	8.2	4.4	5.9
Australasia											
Australia	8.7	5.3	13.3	14.1	17.4	7.0	5.8	9.0	12.7	5.0	4.9
New Zealand [d]	15.3	4.6	7.0	12.6	7.4	9.4	7.3	8.8	11.0	7.7	4.9
Europe											
Belgium											
Czechoslovakia											
Denmark [e]											
Finland											
France											
Germany [f]											
Greece											
Hungary											
Iceland											
Ireland											
Italy											
Netherlands											
Norway											
Spain											
Switzerland											
United Kingdom											
England and Wales	33.0	30.0	52.0	12.7	16.7	14.1	41.8	10.8	19.4	29.6	17.7
Northern Ireland [g]	46.3	36.4	69.3	25.9	32.7	21.4	89.7	20.1	31.0	51.3	34.4
Scotland [i]	25.2	25.3	41.3	12.2	29.5	13.3	54.1	7.9	18.3	37.2	14.6

TABLE I. ANNUAL INFLUENZA MORTALITY-RATES PER 100,000 POPULATION, 1920-51 (continued)

Country	1942	1943	1944	1945	1946	1947	1948	1949	1950	1951
Africa										
Union of South Africa [a]	8.8	11.1	7.1	3.5	2.4	3.5	4.3	3.8	6.6	
America										
Canada [b]	10.5	20.8	15.6	9.0	13.0	8.8	7.1	8.2	6.9	18.4
USA [c, h]	8.1	12.9	13.1	7.7	6.3	5.3	3.5	3.1	4.4	
Chile	63.4	49.7	47.0	43.5	32.8	23.0	24.6	25.6	16.1	
Uruguay	2.6	5.6		13.6	6.6	11.2	8.5	7.0	4.2	
Asia										
Japan	5.4	4.6				2.4	0.6	0.6	1.5	
Australasia										
Australia	7.7	6.1	2.5	2.2	2.8	2.0	4.9	1.5	4.3	..
New Zealand [d]	16.1	4.2	4.0	3.3	6.7	1.9	3.0	2.6	4.3	..
Europe										
Belgium	19.4	18.7	20.2	22.8	21.5	18.5	12.2	14.6	9.8	17.4
Czechoslovakia	3.7									
Denmark [e]	2.9	2.5	5.8	1.7	4.1	3.3	0.7	3.8	6.1	3.4
Finland	9.1	4.9	6.8	2.8	1.4	9.9	2.6	14.0	1.8	8.8
France	4.8	3.9	11.8	5.6	5.9	4.5	3.8	29.3	4.5	19.6
Germany [f]					4.5	6.4	7.1	17.2	7.0	10.8
Greece										
Hungary										
Iceland	1.6	28.8	3.2	0	5.3	7.4	0.7	7.2	3.5	
Ireland	13.1	15.7	27.6	10.4	26.0	21.9	6.7	9.4	10.9	77.7
Italy	9.4	9.1	8.7	6.2	7.9	6.9	11.4	12.5	3.7	9.3
Netherlands	10.2	9.4	15.7	5.6	9.4	5.7	3.5	25.4	6.2	15.7
Norway	2.4	3.0	2.7	1.0	2.5	1.8	1.3	2.0	3.4	2.4
Spain	17.7	15.2	19.2	11.6	7.6	13.4	10.0	8.7	6.4	
Switzerland	7.1	4.6	28.4	13.6	13.0	15.5	5.8	13.2	5.5	24.9
United Kingdom										
England and Wales	8.8	33.3	10.3	7.0	13.0	7.9	2.9	13.0	8.9	36.1
Northern Ireland [g]	15.0	26.0	16.2	10.5	28.6	20.4	5.6	7.1	11.5	64.4
Scotland [i]	6.5	24.8	7.8	4.4	13.9	6.7	2.7	7.7	7.5	21.8

[a] European population only
[b] Excluding Province of Quebec before 1926
[c] Deaths Registration Area, prior to 1933
[d] Excluding Maoris
[e] Excluding Faroe Islands
[f] Federal Republic from 1948
[g] 1941-49, civilian population only
[h] Since 1940, excluding deaths among armed forces outside the USA
[i] 1940-46, civilian population only

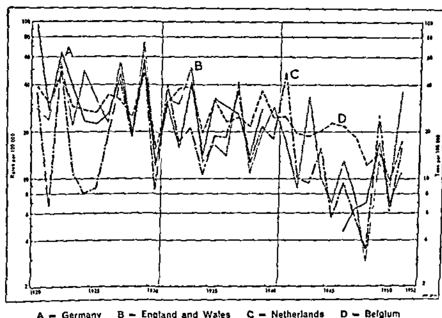
1924, 1927, 1929, 1931, 1933, 1937, and—to a lesser extent—1951, while in the USA and Canada these high levels occurred in 1923, 1926, 1929, 1932, 1937, and 1951. Prior to 1945, low levels of influenza mortality were observed in these countries every two to four years.

Influenza mortality has been falling in all countries for which data are available. This downward trend can be observed both in successive peak years and in the lowest rates of non-epidemic years.

Fig. 3 and 4 represent, on the logarithmic scale, the movement of influenza mortality in various countries. In spite of differences in the range of annual variations, the general trend is apparent.

In England and Wales the downward trend has been observed since 1926 in the non-epidemic years, and since 1929 in the epidemic years. The successive peak-rates during the four epidemic years after 1929—i.e., 1933, 1937, 1940, and 1943—have been decreasing steadily from 73.4 per 100,000 population in 1929 to 33.3 per 100,000 in 1943 (a decrease of about 55% over the fourteen-year period). The fall during the non-epidemic years has been gaining in speed since 1938: the rates, per 100,000,

FIG. 3. INFLUENZA MORTALITY, 1920-51 (I)
(logarithmic scale)



being 10.8 in 1938, 8.8 in 1942, 7.0 in 1945, and 2.9—the lowest on record—in 1948.

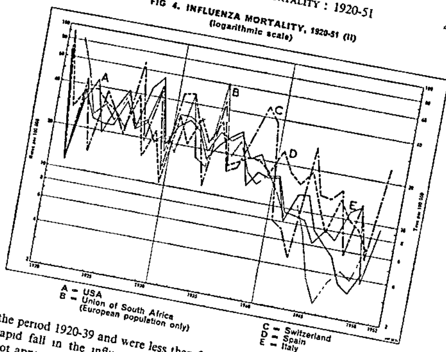
In Germany, the rate of decrease over the period 1920-39 was slower than in England and Wales, the levels during non-epidemic years remaining higher, but the rates in epidemic years being lower.

In Italy, influenza mortality decreased slowly from 1922 to 1937, the rates showing little variation between epidemic and non-epidemic years, except for the 1929 peak and the 1930 low rate. In 1950, the rate fell from 12.5 per 100,000 to 3.7, the lowest recorded so far.

In France, the annual rates show considerable fluctuations due, no doubt, to the tendency to return influenza as a cause of death during an epidemic, rather than to truly high levels of influenza mortality. This view is further supported by the fact that mortality attributed to influenza was lower in France than in other western European countries during non-epidemic years such as, for instance, 1921, 1925, 1930, and 1934. During the post-war years, the rate was again unusually high in epidemic years, 1949 and 1951 (29.3 and 19.6 per 100,000), while it was as low as 3.8-5.9 per 100,000 in 1945-48 and 1950.

In Belgium, the curve shows a slow downward trend with very slight annual variations. Except for the 1929 high (47.4) and the 1930 low (15.3) rate, the annual fluctuations of the rate did not exceed 100% over

FIG 4. INFLUENZA MORTALITY, 1920-51 (II)
(logarithmic scale)



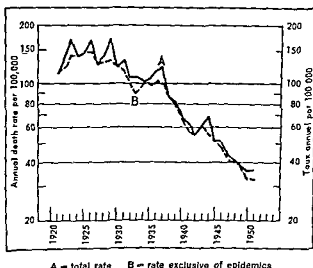
the period 1920-39 and were less than 20% from 1940 to 1947 The recent rapid fall in the influenza mortality-rate observed in most countries is not apparent in Belgium prior to 1948 In contrast to Belgium, the rates show wide annual variations in the Netherlands; the very low rates during known non-epidemic years (1921, 1923-26, 1930, 1945, and 1948) and the high level during peak years suggest that deaths from influenza are reported more frequently during an epidemic

In Switzerland (as might be expected in a smaller country), considerable fluctuation of the annual rates of influenza mortality was recorded throughout the period 1920-51 A decrease in mortality was apparent only after 1940, when rates under 6 per 100,000 were recorded during three non-epidemic years (1943, 1948, and 1950)

In Spain, the deaths ascribed to influenza remained fairly high during the period 1921-35, and reached 32.9 per 100,000 during the epidemic year of 1935 During the following fifteen years, influenza mortality decreased, showing also less variations between high and low annual rates.

The influenza mortality figures for the USA show the slow downward trend with wide fluctuations from 1920 to 1937, and the more rapid decrease of the annual rates after 1937 This striking characteristic of recent trends in mortality caused by influenza is also apparent from the combined influenza and pneumonia mortality-rates

FIG. 5 TREND OF MORTALITY FROM INFLUENZA AND PNEUMONIA IN GROUPS OF CITIES IN THE USA, 1920-51



A — total rate B — rate exclusive of epidemics

Reproduced from Collins & Lehmann [1] by kind permission of the editors of *Public Health Reports (Washington)*

Collins & Lehmann [1] have shown, with regard to groups of cities in the USA, that the acceleration of the downward movement of influenza

up to the middle 1930's, and accelerated during ... was also observed in the Southern Hemisphere, for instance, among the European population of the Union of South Africa (see fig 4), in Australia, and in Chile In the last-mentioned country, where deaths ascribed to influenza were exceptionally high (corresponding to a rate of over 1 per 1,000 from 1920 to 1940), a steady fall in the rate has been recorded since 1933 (from 245.5 in 1933 to 16.1 per 100,000 in 1950).

While the recent fall in mortality ascribed directly to influenza is evident in most countries, the reasons for it cannot be explained fully at this stage The comparatively short period of time corresponding to the accelerated downward movement, prior to the moderate epidemic year, 1951, does not allow of a conclusion to be drawn as to whether influenza mortality has reached its lowest level in non-epidemic years. It is also likely that virus identification practised on a wider ... has resulted in fewer deaths

being returned as due to influenza when the virus is known not to have been identified.

All influenza epidemics invariably result in an increase in general mortality, and in mortality from various causes, and, as stated earlier, the excess mortality during the period of the year corresponding to an epidemic provides an index to the resulting loss in human lives

The effect of an epidemic on mortality can also be seen from a comparison of annual death-rates during epidemic and non-epidemic years. While such rates for various countries are not directly comparable, it is of interest to note, for instance, the fall in mortality levels in five countries from 1937, the last pre-war epidemic year, to 1938, a non-epidemic year (see table II) The fact that the percentage decrease in death-rates from specified causes (except from bronchitis in Canada and Sweden, and pneumonia and bronchopneumonia in Canada) is higher than that from all causes, shows the effect of an epidemic on the causes of death during 1937. The excess mortality from various causes is, of course, still more apparent in the study of data limited to the epidemic period Martin,[5] who studied the mortality in England and Wales (in the epidemic years 1922, 1924, 1927, 1929, 1931, 1933, and 1943), found in the quarter corresponding to the epidemic, as compared to the same quarter of the previous year, an excess of deaths from 13 4% to 41.8% for the respiratory diseases, from 13% to 27.2% for the circulatory diseases, and from 1.8% to 3.3% for tuberculosis

The figures for England and Wales do not indicate any significant shift, in successive epidemic years, in the excess mortality from the above-mentioned causes In view of the recent fall in mortality ascribed to influenza, an analysis of data for certain countries would be of interest in order to determine whether there has been a true decline in the mortality due to influenza or whether deaths from this disease are now charged to other causes. In their remarkable study relating to groups of cities in the USA, Collins & Lehmann[2] have shown that influenza and pneumonia account for a lesser share in the excess mortality of recent epidemics than was the case previously, while the proportion of deaths attributed to other causes, and particularly to non-respiratory chronic diseases, has correspondingly increased (see table III). Heart disease was the larger non-respiratory contributor to these excess deaths

An important aspect of the changes in mortality directly charged to influenza during successive epidemic years is the gradual drop in the number of deaths in the lower age-groups, observed in most countries after the 1918-19 epidemic. Logan[3] noted in respect of England and Wales that the proportion of influenza deaths at ages under 55 years was 86% in 1918, 75% in 1919, 37% in 1929, 41% in 1943, and only 11.5%[a] in 1951 A decrease in the influenza mortality-level has, naturally, been

[a] Revised figure

TABLE II. MORTALITY BY SPECIFIED CAUSES IN FIVE COUNTRIES IN 1937 AND 1938 (RATES PER 100,000 AND DECREASE FROM 1937 TO 1938 IN %)

		Canada	USA	England and Wales	Netherlands	Sweden
All causes	1937	1,024 9	1,122,1	1,241 9	878 3	1 201 3
	1938	959 2	1,064 0	1,161 8	852 6	1,154 4
	%	—6 4	—5 4	—6 2	—2 9	—3 9
Influenza	1937	47 4	29 4	45 4	36 7	18 7
	1938	21 2	12 7	11 8	12 7	3 0
	%	—32 8	—56 8	—74 0	—65 4	—84 0
Bronchitis	1937	3 0	3 1	43 6	15 1	8 3
	1938	2 9	2 8	32 2	13 5	8 2
	%	—3 3	—9 7	—26 1	—10 6	—1 2
Pneumonia and broncho-pneumonia	1937	69 6	85 1	72 0	62 5	104 1
	1938	66 7	67 7	63 0	58 3	93 9
	%	—4 2	—20 4	—12 5	—6 7	—9 8
Other disease of the respiratory system	1937	14 7	8 2	11 8	12 5	11 6
	1938	13 7	7 7	10 1	10 2	10 1
	%	—6 8	—6 1	—14 4	—18 4	—12 9
Causes of death unstated or ill-defined	1937	7 0	15 9	3 0	33 9	4 5
	1938	5 5	15 1	2 6	32 6	3 8
	%	—26 7	—5 0	—13 3	—3 8	—15 6

recorded in all age-groups, but this decrease is much slower in the higher age-groups Martin[4] has shown in respect of England and Wales that the influenza death-rate decreased between the period 1920-24 and that of 1940-44 by 75%-80%, at ages 5-35 years, and by 40%-70%, at ages above 35 years.

The gradual disappearance of influenza as a certified cause of death, and the shift of excess mortality to causes other than influenza and pneumonia, have, no doubt, resulted in various countries in a further shift of mortality due indirectly to influenza epidemics towards the higher age-groups.

* * *

The appraisal of the importance of total morbidity and mortality caused by influenza is difficult owing to the lack of satisfactory statistical data concerning this disease The mortality caused by its epidemics may, however, be estimated from a computation of the excess of the general mortality during epidemic periods, as compared to similar seasonal periods in non-epidemic years

TABLE III. EXCESS MORTALITY PER 100,000 FROM ALL CAUSES AND FROM INFLUENZA AND PNEUMONIA DURING THE WHOLE OF EACH EPIDEMIC FROM 1918 TO 1951, IN GROUPS OF CITIES IN THE USA, AND PERCENTAGE OF EXCESS MORTALITY FROM ALL CAUSES CREDITED TO INFLUENZA AND PNEUMONIA *

Epidemic	All causes	Influenza and pneumonia	Percentage
1918–19	598 0	550 5	92
1920	125 5	97 2	77
1928–29	64 9	40 8	64
1930–31	57 1	30 2	63
1922–23	50 4	32 3	61
1943–44	48 8	14 4	58
1926	48 3	28 2	53
1932–33	45 7	22 2	50
1936–37	41 6	20.8	49
1935–36	38 9	15 5	45
1931–32	36 1	13 8	40
Early 1922	34 1	20 7	38
Spring 1928	31 8	14 5	34
1939	26 3	5 5	33
1945–46	24 4	3 7	29
1934–35	18 4	6 3	26
1947	16 6	2.5	21
1940–41	15 7	5 2	19
1951	15 7	4 1	17
1950	14 5	2 7	15
Early 1940	14 5	2 5	15

* After Collins & Lehmann [5]

The curves of mortality directly charged to influenza in most countries for which data are available for the period 1920-51 show a gradual decrease in the range of annual variations and a fall in mortality accelerated after the middle 1930's

The short period during which this more rapid fall has been apparent does not allow of stating whether influenza, as a certified cause of death, will continue its downward trend in future non-epidemic years, or has already reached its lowest level.

Both influenza and pneumonia, and the associated chronic non-respiratory causes of death, have decreased in the successive epidemic years, however, the latter have done so at a lower pace and their relative proportion has become accordingly higher, which accounts for a slower

decline in the mortality recorded for the higher age-groups in recent epidemics.

There seems to be no doubt about the effect of modern therapy in decreasing mortality from infections associated with influenza and particularly pneumonia.

It is likely, however, that part of the impressive decline observed in mortality directly charged to influenza is due to more precise diagnosis of the actual causes of death, and possibly to a change in the rules governing the selection of the underlying cause when several are mentioned on death certificates.

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THE INFLUENZA VIRUS : ITS MORPHOLOGY, IMMUNOLOGY, AND KINETICS OF MULTIPLICATION

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I

Morphology of Influenza Virus

Influenza virus consists of at least two distinct components, the elementary body and the soluble substance [48, 52] By high-speed centrifugation of influenza virus suspensions the former particles are easily sedimented while the latter remain suspended in the supernatant fluid. The properties of the two components differ considerably.

The soluble substance consists probably of small particles with an average diameter of approximately 10 mμ [36, 54, 75, 79] These particles are not infective, they are not adsorbed on red cells, and they do not cause haemagglutination. The soluble substance can be demonstrated only by complement-fixation tests in which it acts as a powerful antigen Antibodies to soluble substance develop, as a general rule, only after infection, not after vaccination [79]

The elementary bodies are considerably larger than the soluble substance On the basis of centrifugation [29, 53, 71] and filtration [21, 29] data their size has been estimated to vary from 80 mμ to 120 mμ. Studies on the properties of the elementary bodies have shown that virus infectivity is intimately associated with these bodies; furthermore, the elementary bodies react with red cells and cause haemagglutination Moreover, they can immunize animals which develop virus-neutralizing and other antibodies, and become resistant to infection. The elementary bodies also fix complement in the presence of immune serum, but the complement-fixing antigen is apparently different from that associated with the soluble substance [52]

Because of its small size the soluble substance is not easily seen, even with the best electron microscope. For this reason almost all studies of the morphology of influenza virus have been concerned with the elementary bodies and associated larger forms of the virus.

In electron-micrographs of infected allantoic fluid purified by centrifugation or by adsorption of the virus on red-cell ghosts,[18, 19] the elementary bodies are seen as round or slightly ovoid particles measuring 80-120 mμ in diameter, the average size of influenza B virus (Lee strain) being somewhat larger than that of influenza A virus (PR8 strain) [18, 53, 70] In addition, elongated forms have been observed either as long filaments (1 μ or more in length) or as rods of intermediate length.

The number of filamentous forms in infected allantoic fluid has been found to vary considerably from one strain to another. Fluid from eggs infected with old laboratory strains, such as PR8 and Lee, usually contains only a few filaments, by contrast, in fluids infected with more-recently isolated A virus (FM1 and related strains) the number of filamentous forms is often comparable to the number of elementary bodies.[15] However, recent studies of intracellular virus growth have shown that under suitable conditions large numbers of virus filaments, together with spherical elementary bodies, are produced in infected tissues by all strains of influenza examined.[20, 63, 64, 80] The length of the filaments has been found to vary from strain to strain; in swine influenza, for example, the filaments are rather short, while recently isolated A strains show very long filaments [64] In the latter strains the prevalence of filamentous forms also appears to be greater than in the old PR8 and Lee strains, but, as mentioned above, this difference is not as marked in infected tissues as it is in allantoic fluid.

It is now generally assumed that the filamentous structures described above represent a form of virus This assumption is substantiated by several observations In the first place, they are not normal cell components for they have never been seen in the normal allantoic fluid of 10- to 13-day-old eggs [15] Although filaments may be seen in normal cells it is possible to differentiate them by their structure from virus filaments.[63] Next, virus filaments are not artefacts produced by drying or other handling procedures before examination under the electron microscope, for they may be observed directly in untreated allantoic fluid with a darkfield microscope.[15, 45] Furthermore, the filamentous forms react with red cells in the same way as do the elementary bodies, being adsorbed onto, and eluted from, the red-cell membrane. After heat-inactivation both forms of virus may be adsorbed together on the red-cell membrane, but they do not elute spontaneously. Addition of homologous antiserum, however, removes both long and round forms from the membrane, while heterologous serum has no such effect Finally, studies using the darkfield microscope have shown that homologous serum agglutinates the filaments whereas heterologous serum causes only slight or no agglutination [15]

While the above results strongly suggest that these filaments represent a form of influenza virus, their exact relationship to the spherical virus is still incompletely understood Evidence has been presented which indicates that the elementary body can give rise to the elongated forms when

grown in a suitable medium [15] Moreover, the ratio of filaments to spheres has been found to be fairly constant at various stages of the infection, indicating that filaments and spheres are continuously produced.[63]

It has been suggested that the filamentous forms may represent a stage in virus multiplication [15] and that at least some of the spheres may arise by segmentation of the long forms. This suggestion is based on micrographs showing gradations between unsegmented filaments, segmented rods, beaded chains, and spheres [20, 60, 63, 64] As another possibility it has been suggested that the filaments consist of masses of aggregated virus protein linked by tubes of lipoid material derived from the cell wall.[45] The evidence concerning both hypotheses is not conclusive, and the question of the relationship between the elongated and spherical forms of influenza virus must await further experiment.

Immunology of Influenza Virus

The strains of human influenza virus known at the present time can be divided into three distinct and immunologically unrelated types, which are referred to as influenza A, B, and C. In addition, there are the swine influenza viruses, which are serologically related to the influenza A viruses. The knowledge of the antigenic structure of influenza C virus is still limited, whereas there is a wealth of data concerning the antigenic constitution of influenza A and B viruses

As described above, it has been shown that influenza virus suspensions contain at least two different virus components, the elementary body and the soluble substance [48] These two components differ in antigenic structure. Apparently the soluble substance contains only a single antigen which is type-specific, i e., all strains of influenza A virus contain the same soluble antigen, which is quite distinct from that present in influenza B virus strains. [79]

In contrast to the soluble substance, the elementary bodies and probably also the elongated fo[illegible]
ponents, and it is a [illegible]
the same serological [illegible] s
regards antigenic constitution. Thus, although all influenza A strains are immunologically related, marked differences in the antigenic pattern of individual strains can be easily demonstrated in cross-tests with different virus strains and their antisera

Similar variations have been recognized also among individual strains of influenza B virus, whereas so far only one serological type of influenza C is known.[25]

Comparisons of the antigenic pattern of influenza virus strains can be carried out by a number of methods such as cross-neutralization-tests in mice,[25, 73] cross-protection tests in mice,[24, 73] and haema[illegible]

inhibition [11, 12, 37-41, 65, 68, 69, 78] and complement-fixation tests [27, 31] using elementary-body suspensions as antigen. In recent years the haemagglutination-inhibition test has been preferred by most workers because this method is technically the simplest, and because it is less expensive than most of the other tests.

Differences in antigenic pattern between two strains may be due to quantitative as well as qualitative variations in the antigenic composition. In the former case both strains contain the same antigenic factors but in different proportions; in the latter case each strain contains antigenic components which are not present in the other. A distinction between quantitative and qualitative antigenic variation is possible in studies of virus-antibody interaction with specific antisera which have been absorbed with homologous and heterologous virus strains.[28, 44] Results obtained in such experiments indicate that the antigens associated with the elementary bodies of influenza A strains may be divided into group-specific antigens which are responsible for the A-character, and type-specific antigens which are peculiar to certain groups of strains.[44] So far, however, only a few strains have been studied by the absorption method, and much more work must be carried out before it is possible to attempt a classification of influenza viruses by this method. It seems very probable that tests with absorbed sera may furnish the best approach to the solution of this problem

On the basis of results obtained in reciprocal cross-tests between large numbers of different influenza virus strains and their antisera, attempts have been made to segregate the virus strains of each serological type into subtypes. However, the strains of the various subgroups tend to merge into one another, and a precise subgrouping has not been possible. The problem is further complicated by the fact that results obtained in different laboratories have been somewhat conflicting This may—to some extent at least—be ascribed to differences in techniques employed by various workers Furthermore, the use of immune sera from different animal species may also influence the results [39, 61] Finally, it has been shown that antigenic variations may be induced by serial passage of influenza virus strains in the laboratory.[25, 43] Although this latter fact may be of less importance than originally assumed,[44] it is now recommended that the antigenic constitution of strains be studied with virus which has been through only a few passages in the laboratory.

In spite of the somewhat controversial results obtained in various laboratories, and in spite of the fact that there is as yet no generally agreed standard procedure for antigenic analysis of influenza virus strains, there is nevertheless a satisfactory degree of concordance as regards classifica-

Similarly, the B strains so far examined can be subdivided into two groups with the general characteristics of Lee (1940) and Bon (1943) virus[11, 12]

From a practical point of view the system of classification outlined above is very useful. It is simple and rapid and, by including more prototype strains, it can easily be extended if new antigenic subtypes should appear. In this connexion it should be mentioned that some workers have already selected as a fourth influenza A prototype a strain isolated in 1950 (FW-1-50). In addition, they have worked out antigenic formulae for a number of strains on the basis of the extent of reaction of the virus under study with the antisera to the prototype strains.[38, 39]

Variations in antigenic pattern of influenza virus isolated since 1933

Studies of virus strains isolated in recent years have indicated that in most epidemics only one antigenic type was incriminated. Furthermore, a notable homogeneity has been observed among strains isolated from widely separated areas of the world during the same year. The impression is gained that in recent years the antigenic variations among influenza viruses have been small and continuous

However, in certain periods major shifts in antigenic constitution seem to have occurred Such a happening apparently took place in 1946-7 with the appearance of virus strains of the FM1 (1947) type (the so-called A-prime group), which had not been encountered before. Furthermore, it seems possible that from 1933 to 1940 a wide variety of strains have been prevalent at the same time, even in the same epidemic. The apparent heterogeneity of strains isolated before 1940 is, however, not definitely proved, because these strains were all isolated in ferrets and adapted to mice, and thus have possibly been altered through animal passage[13, 61] Further studies of freshly isolated strains from future epidemics are therefore required in order to obtain precise information regarding the overall epidemiological picture of influenza.

Studies of the antigenic variations among influenza viruses are of importance not only for a better understanding of the epidemiology of the disease, but also for problems associated with the procuring of an effective vaccine against it. It has been clearly established that a vaccine made from strains which differed considerably from the epidemic strain in question did not protect against the disease[26] However, the significance of minor variations upon vaccine effectiveness has not been clearly proved. To solve this problem, more studies on the importance of minor strain differences for the development of immunity are required, and the results obtained in such in vivo tests should be compared with those obtained in haemagglutination-inhibition tests. Few attempts of this kind have been made so far.[40]

The mechanism responsible for the changes in the antigenic pattern of influenza virus cannot be satisfactorily explained at present. One pos-

sibility is that influenza viruses are constantly mutating, and that the form prevalent during a certain period of time disappears to give place to entirely new antigenic types[5] However, it appears equally possible that the influenza viruses contain a large but finite number of antigenic components which may be present in different combinations and in different proportions.[66] Changes in antigenic pattern may be the result of selection or rearrangement and recombination processes induced by environmental factors such as passage in partially immune human hosts. That this latter factor may be of importance is substantiated by experimental evidence showing that persistent antigenic variants could be produced in the laboratory by growing the virus in chick embryos in the presence of heterologous immune serum.[7, 8]

Variations in serological behaviour of strains

Certain variations in the serological behaviour of influenza virus strains are caused by factors other than differences in antigenic constitution.

Non-specific inhibitor. It is a well-known fact that most animal sera contain non-specific substances which combine with virus and inhibit haemagglutination[41, 67, 78] The concentration of inhibitor varies from

Haemagglutination-inhibition tests with sera containing large amounts of inhibitor may therefore lead to erroneous results. However, the inhibitor substance may readily be destroyed by *Vibrio cholerae* filtrate, whereas the specific antibodies are not seriously affected by such treatment.[15, 50, 62] The difficulties caused by non-specific inhibitor may thus be abolished in a rather simple way.

Avidity effect. In haemagglutination-inhibition tests some antisera have been found to inhibit heterologous virus strains to higher titre than homologous strains. These paradoxical results have been ascribed to differences in the combining-power or "avidity" of the virus strains for antibody. Thus, it has been shown that some virus suspensions needed a large amount of serum per unit of haemagglutinin for inhibition; such preparations were said to have a high avidity. Conversely, virus suspensions requiring only small quantities of serum per unit were said to have a low avidity.[42]

In recent years three different types or phases of avidity variations have been described[51, 75] The characteristics of these three phases (P, Q, and R) are as follows P-phase virus is inhibited to high titre by its homo-

logous serum only Q-phase virus is poorly inhibited by all sera including the homologous one, even though it stimulates the formation of antibodies which react in high titre with P-phase virus. R-phase virus, finally, is inhibited to high titre both by homologous and by related antisera.

The avidity differences appear to have the character of an unstable phase variation. It is possible in the laboratory to convert a P-phase strain into Q-phase by cultivating the virus in the allantoic cavity in the presence of heterologous antiserum. Conversely, Q-phase virus can be altered to P-phase by mouse-lung passage.[19, 56]

Avidity differences may add to the difficulties involved in studies of the antigenic relationship of influenza virus strains, and may necessitate the introduction of correction factors.[42] On the other hand, avidity differences have been found useful in recent epidemiological studies of influenza. In 1950-1 a large number of influenza A strains were collected from various countries. These strains were studied at the World Influenza Centre and it was demonstrated that all strains examined belonged to either of two antigenically slightly different groups, the "L" (Liverpool) and the "S" (Scandinavian) subgroup. All L forms were found to be in the P phase, i e., they were inhibited to high titre by homologous-type serum; whereas almost all S forms were in the Q phase and were poorly inhibited by homologous serum. By a simple avidity test with homologous-type antiserum it was thus possible to classify the strains examined as belonging to either the L or the S subgroup.[51]

Knowledge of the mechanisms responsible for the avidity phenomenon is still incomplete and the problem requires more attention. It should be mentioned that avidity effects have been observed not only in haemagglutination-inhibition tests but also in neutralization tests in mice and eggs [51] The phenomenon appears to be pronounced only in tests with sera from ferrets and has not been observed when chicken sera have been employed [39 51] It should also be added that, generally speaking, Q strains have been found to produce poorer antisera in the ferret than P and R strains [78] This latter fact may be of importance for the selection of strains for incorporation in influenza virus vaccines.

Kinetics of Influenza Virus Multiplication

Human influenza virus is capable of multiplying in several different animal hosts, e g, ferrets,[74] mice,[6] and hamsters,[76] as well as in a number of other animal species. Virus may be cultivated also in the developing chick embryo,[72] in de-embryonated eggs,[9] and in tissue-cultured chick embryo.[83] Detailed studies of influenza virus multiplication have been carried out in mice,[17, 45, 77] and to an even greater extent in fertile eggs [33 45 47, 65, 57 39]

Virus growth in the mouse lung

After intranasal inoculation of mouse-adapted virus in mice, the virus content of the lung remains fairly constant for from five to six hours. Then follows a period of rapid increase of virus, and maximum titres are reached 24-48 hours after inoculation, depending on the amount of virus administered. The titre remains high until the sixth day and then decreases, probably because of antibody formation. In mice which have received a lethal dose of virus, the titre levels ultimately reached are independent of the amount of virus inoculated. When sublethal doses are administered, the virus growth-curve parallels the curve obtained with lethal doses; the maximum titres are, however, about a hundredfold lower than in animals treated with a lethal dose of virus.[77]

Similar studies with virus strains which were not adapted for mice have led to somewhat conflicting results. Some unadapted strains have been found to multiply readily in the mouse lung and, although they did not produce any lesions, the virus content of the mouse lung was as high as that obtained with adapted strains.[41] This observation suggested that adapted and unadapted strains differ in their pathogenicity for the mouse rather than in their ability to multiply in the mouse lung In contrast, other unadapted strains have been found to achieve final titres which were inferior to those obtained with mouse-adapted virus, indicating that in this case the adapted virus was a more efficient parasite than the unadapted one.[17]

Virus growth in the developing chick embryo

Virus may be propagated by a number of different methods in chick embryos. A detailed description of these methods may be found in a monograph by Beveridge & Burnet.[10] Among the various chick embryo techniques two have proved of particular value in the study of influenza virus: (1) the amniotic method for isolation of virus from human throat washings, and (2) the allantoic method for the production of large amounts of virus and for titration of influenza virus in eggs. For studies of the kinetics of influenza virus multiplication, the allantoic method has also proved of great value, and the bulk of present knowledge concerning this problem has been procured in studies with chick embryos inoculated in the allantois.

The growth of influenza virus after intra-allantoic inoculation is at first localized to the allantoic membrane. Later on, the virus reaches all parts of the egg—probably through the blood-stream, but this has not been studied in detail. The susceptibility to infection is practically the same in eggs from different kinds of hen and in eggs of different ages. For practical purposes, however, it has been found convenient to use eggs which have been incubated for 10-11 days before inoculation.

Studies of the virus growth-curve in the allantoic membrane and fluids have shown that the infective titre remains fairly constant during the first five or six hours after inoculation of influenza A virus. The constant period of influenza B virus varies between six and ten hours according to the amount of virus inoculated [55] In the ensuing hours there is a rapid increase of virus, and maximum titres are reached at 12-48 hours after inoculation, depending on the concentration of the seed virus During the period of rapid increase of virus the infectivity and haemagglutination titres usually parallel each other, but later on the infectivity titre decreases, probably because of heat-inactivation of the virus, whereas the haemagglutination titre remains fairly constant for several hours

The influence of various experimental conditions on influenza virus multiplication in the allantois has been studied and two factors have been found to be of some importance—the temperature of incubation, and the concentration of seed virus employed [57, 59]

Effect of incubation temperature Influenza A virus (strain PR8) has been found to attain comparable titres in eggs incubated at temperatures ranging from 30° to 39° C; however, the rate of increase of virus is considerably higher at 37°-39° C than at the lower temperatures [57] The optimum temperature for influenza B virus appears to be somewhat different This virus has been found to grow better at 35° C than at 37° C and it often fails completely to grow at 39° C [56]

Effect of the concentration of the seed virus. As mentioned above, the rate of increase of virus varies with the amount of virus inoculated. With large doses of seed virus, maximum titres are reached within 12-15 hours after inoculation, whereas, with weak inocula, the peak of virus activity is not attained until 48 hours after inoculation. Comparison of the titre levels ultimately reached has shown that the total amount of virus produced—as measured by haemagglutination titration—is virtually the same, regardless of the concentration of the seed virus However, the infectivity titre in eggs inoculated with large doses of virus tends to be slightly inferior to the titres attained in eggs infected with dilute virus. This apparent dissociation between infectivity and haemagglutinating activity has been found to be even more pronounced in de-embryonated eggs,[9] and in embryonated eggs it became more marked on continued passage of undiluted allantoic fluid by the allantoic route.[58] Under such circumstances large amounts of haemagglutinating but non-infective virus ("incomplete" virus) was produced, which was found to interfere with and inhibit multiplication of infective virus. In this connexion it should be mentioned that similar "incomplete" forms of virus have been obtained in mice after intracerebral inoculation of large doses of virus [60] So far no satisfactory explanation of the nature of the incomplete forms of virus has been presented

Influenza-virus/host-cell interaction

The entodermal cells lining the allantoic cavity are arranged in a single layer, and the number of cells present can be estimated with some accuracy.[14, 16, 31] This comparatively simple host-cell system has been found of particular value for analyses of influenza virus/host-cell interactions. Results obtained in such studies[14, 35] indicate that the virus growth occurs in one or more successive cycles of reproduction, and that the processes involved in each of these cycles may be divided into at least four major steps, comprising (1) the adsorption of the virus onto the host cells; (2) the entrance of the virus into the cells; (3) the multiplication of the virus within the cells; and (4) the liberation of the newly formed virus from the host cells. After release into the allantoic fluid, the virus may then attack other susceptible cells of the allantoic sac and thus initiate a new growth cycle.

The various attempts to analyse further the individual steps of the infective process[14, 22, 30, 33, 35, 46, 47] will not be considered here. However, it seems of interest to discuss briefly some experiments which support the general assumption that some host-cell metabolic systems are required as the energy source for the reproduction of virus. As regards influenza virus, this concept is to some extent supported by observations on the effect of large doses of irradiated virus in the allantoic cavity After the inoculation of such virus the growth of the allantoic cells is inhibited,[34] and they become resistant to infective influenza virus. Both these observations can be explained if it is assumed that the irradiated virus interacts with enzyme systems within the host cells which are essential for the reproduction of virus as well as for the proper functioning of the cells.[32] Interference between two active viruses may be explained in a similar way, i.e., as competition between the opposing agents for certain intracellular enzyme systems of the host cells.

More indirect information concerning the significance and nature of the metabolic systems of the host cells which are necessary for influenza virus multiplication has been obtained in recent years, in studies with virus grown in tissue cultures and in de-embryonated eggs. Evidence has been presented which indicates that under these conditions the oxidative processes of the citric-acid cycle are essential for the synthesis of the virus[1, 16] This observation has been confirmed in infection experiments in mice in which the citric-acid cycle was partially blocked by the administration of sublethal doses of sodium fluoracetate.[3] In such animals the growth of influenza A virus was significantly inhibited. Other studies have shown that methionine[2] and certain other amino-acid metabolites normally present in the susceptible host cell are also involved in the biosynthesis of influenza virus.[4]

The recent advances in knowledge of the enzymatic virus/host-cell interactions appear very promising, and it seems likely that further studies

of this kind may not only provide a better understanding of the kinetics of intracellular virus growth but also lead to results of the greatest value as regards the control of influenza by chemotherapy

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RECENT WORK ON THE INTRINSIC QUALITIES OF INFLUENZA VIRUS *

Somatic and Genetic Aspects

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Over the past seven or eight years there has been a continuous programme of work at the Walter and Eliza Hall Institute on what may be called the intrinsic qualities of influenza viruses, as opposed to work more directly concerned with the relation of the virus to clinical and epidemiological aspects of influenza Out of this work there has emerged the concept of the virus particle as a twofold entity composed, like any other living organism, of somatic and genetic constituents. As elsewhere in biology, the properties of the soma can be studied by what may be termed physiological and biochemical methods, while those of the genetic apparatus (which for convenience we shall term the "genome" as a single word to balance and contrast with "soma") are studied wholly at the genetic level All phenotypic characters are expressed in the soma and, just as a higher organism is built wholly from the zygote, the soma of the influenza virus is reconstructed by the genome at each generation

SURFACE (SOMATIC) QUALITIES OF INFLUENZA VIRUSES

Broadly speaking, all the characters by which influenza viruses can be differentiated in the laboratory are based on surface qualities—serological character, activity as haemagglutinin and enzyme, and adsorption to the surface of susceptible host cells Only the intensity of the damage provoked in infected cells can be regarded as something not directly associated with surface aspects of the virus

Serological aspects of influenza viruses are discussed elsewhere in this monograph [a] In the present connexion all that need be said is that there has been no work to indicate the chemical nature of the surface antigens of influenza viruses It is known that in the presence of antibody, adsorption to the red-cell surface fails to occur, but this does not necessarily mean that the adsorptive patterns to be discussed here are the

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[a] See pages 25 and 87

actual antigenic determinants. Like every other inheritable quality, antigenic behaviour is controlled by the genome. Recent work by Hirst [40] suggests strongly that genetic changes in antigens may represent the sharp replacement of one genetic determinant by another.

The interaction between virus and cell surface has been studied chiefly in relation to haemagglutination, but there is adequate evidence to justify the statement that all the essential findings established in regard to the red-cell surface are equally applicable to the surface of susceptible cells, including those of the mammalian respiratory tract and those lining the allantoic cavity of the chick embryo.

Haemagglutination

In 1941, Hirst [36] and McLelland & Hare [45] showed independently that infected allantoic fluid would agglutinate chicken red-cells. Within the next few years it was shown clearly that the agglutinating agent was the virus itself (Friedewald & Pickels [33]). A minor qualification was subsequently introduced as a result of von Magnus's [47] work on "incomplete" virus Under certain conditions, when a large amount of virus is used to inoculate chick embryos in the allantoic cavity, the fluid obtained 21 hours later has an infectivity·haemagglutinin ratio much lower than is obtained when a very small infecting dose is used In the first type of fluid it is assumed that up to 90% of the virus particles are incapable of initiating infection although having the morphological, serological, and adsorptive qualities of normal virus.

With fully active virus fluids there is a constant relation between the 50% infectivity end-point (ID_{50}), as measured by allantoic infection, and the minimal agglutinating dose (AD) of a given fluid. With the techniques used in Melbourne, a fluid with a haemagglutinin titre of 100 will show $10^{8.2}$ ID_{50} per ml One AD = $10^{6.2}$ ID_{50} (Cairns, Fazekas de St. Groth & Edney [21]).

When viruses of the influenza group react with red cells the one thing they have in common is that union with the cell surface occurs, and if the virus is in sufficient quantity—i e, more than about one virus particle to every ten red cells—haemagglutination will be detectable by an altered sedimentation pattern More-detailed study will show that a variety of different interactions occur If we include Newcastle-disease (ND) and mumps viruses in the group, the following phenomena can be observed under appropriate conditions · (*a*) haemagglutination, (*b*) elution of virus with stabilization of the cells and loss of the "receptors" concerned; (*c*) the receptor gradient effect—i e, when, after treatment with one virus, cells have lost their susceptibility to agglutination by that virus, they may remain agglutinable by other viruses of the group; (*d*) diminution in electrophoretic mobility (EPM) after virus action, (*e*) modification of antigenic

character after virus action—uncovering of T-agglutinogen (see page 71); (*f*) irreversible union of virus to cell surface, demonstrable by the capacity of treated cells to agglutinate normal cells and to be agglutinated by anti-viral sera; (*g*) haemolysis by ND and mumps viruses.

Each of these phenomena will require some comment, but it is desirable first to say something about the virus enzyme which is the central feature of most of the phenomena. In 1942 Hirst [37] observed that if virus were allowed to act on red cells for some hours at 37°C the initial agglutination disappeared, the virus was liberated into solution again, and the cells, when washed and tested, were no longer agglutinable by the virus. The re-liberated virus was still fully capable of inducing the same cycle of adsorption, agglutination, elution, and stabilization with fresh red-cells, and Hirst at once suggested that the virus was in this respect behaving like a typical enzyme whose substrate was a surface component on the cell, conveniently termed receptor substance. This concept has been fully established by later work (reviewed by Burnet [9]). Without elaborating the steps by which the conclusions were reached, it can be said that the viruses of the group carry, as part of their surface, chemical groups with enzymic function which have as substrate carbohydrate-containing groups of a variety of mucoproteins and (probably) mucopolysaccharides. A soluble enzyme acting on precisely the same substrates can be obtained from *Vibrio cholerae* cultures and concentrated to a relatively high state of purity (Ada & French [1]). Both viral enzyme and the soluble enzyme—receptor-destroying enzyme (RDE)—in addition to modifying red-cell surfaces, act readily on soluble mucoproteins, the effect being demonstrable (*a*) by destruction of their capacity to inhibit haemagglutination by "indicator" virus (see pp. 71-72), (*b*) by change in their electrophoretic behaviour (Ada & Stone [4]), and (*c*) by the splitting-off from the mucoprotein of an isoglucosamine-peptide unit (Gottschalk [33]).

Standard action of influenza viruses on red cells

We can probably visualize the red-cell surface as a mosaic of lipids, proteins, and mucopolysaccharides, and as far as the influenza virus reactions are concerned, we can think of a loose mesh of macromolecules of mucopolysaccharide covering the whole area of the cell surface and integrated with the other components of the surface. In view of the general character of mucopolysaccharides, there may well be loose strands of macromolecules projecting from the surface as well.

The first aspect of adsorption requiring comment is the necessity for ions to be present in the system (Burnet & Edney [16]), the greater effectiveness of Ca^{++} ions suggesting that the cations are primarily involved. In line with the hypothesis of Puck, Garen & Cline [48] for the adsorption of 'phage to host bacteria, we may postulate that the primary adsorption of virus to red cell is an electrostatic one by which the reactive centres,

on virus and cell surface are brought into primary unoriented apposition. Everything points to this primary adsorption as being mainly responsible for haemagglutination Since the enzymic action of virus or RDE renders the cell incapable of adsorbing virus, it is natural to suppose that adsorption is primarily a function of enzyme and substrate, or more precisely of the active grouping, E, of the enzyme and the corresponding attachment groups of the substrate. In our view (Burnet [10]) the phenomena are best explained by the assumption that, in the presence of appropriate cations, E and S groups are so charged that there is a substantial electrostatic attraction between them. This will be sufficient to hold any virus particle to the cell surface by a number of E-S bonds and, where more than one cell is involved, to form a bridge by which cells can be held together and agglutinated. Once such a series of primary unions has been effected, opportunity is available for mutual orientation of E and S which will allow short-range forces to come into play. The most usual result is enzymic destruction of S with breaking of the corresponding bond holding virus to cell surface. Ada & Stone [4] have provided direct evidence that a single virus particle can, as it were, " browse " over the surface of the red cell As one E-S bond is dissolved, opportunity to make another arises; the virus is continually destroying receptors, but is always held by a sufficient number of bonds to maintain it on the cell surface.

The interaction between virus enzyme and its substrate is complicated by the fact that both agents are on the surfaces of relatively massive carriers and are built into complex molecular patterns which provide opportunity for various types of interference. The soluble enzyme RDE appears to be able to remove all, or virtually all, the S groups on the cell surface. Any virus, however, finds it possible to remove only a more or less standard proportion, presumably because effective contact with the remaining S groups is prevented by interference between adjacent molecular groupings. With an occasional minor exception, the viruses can be arranged in a linear order—referred to as the receptor gradient by Burnet, McCrea & Stone [22]—such that viruses later in the gradient can still agglutinate cells treated by, and rendered inagglutinable by, viruses earlier in the gradient The receptor gradient can be demonstrated more conveniently by treating cells with graded amounts of RDE and stopping the action after a standard time. This gives virtually the same order, with the important exception that mumps virus comes much later in the RDE gradient—it is first if viruses are used A very useful index of the change induced in red cells by these enzymes is given by the change in EPM With human cells which normally have an EPM of 1.30 μ per second per volt per cm, full action of RDE reduces the value to 0.17. In a series of papers, Ada & Stone [4, 51, 55] have described a detailed study of this phenomenon The results are too complex for discussion in this review, but they lead to the very interesting conclusion that there are two

series of receptors on human cells, of which only one is available for the agglutination action of the viruses normally used in this work. The other series is demonstrable by its contribution to the EPM; these receptors can be removed by RDE and by two viruses—ND, and swine influenza. Recent work by White[59] strongly suggests that agglutination by influenza C virus is mediated by a small fraction of receptors in this second series.

An important finding made early in the work on haemagglutination was that cells treated with virus and stabilized were agglutinable by low dilutions of any type of serum (Burnet, McCrea & Stone[13]). The recognition that this work was identical with Thomsen's[58] phenomenon of "pan-agglutination" led to the discovery of RDE. The agglutinin in normal human serum responsible for this action is usually known as "T-(Thomsen-) agglutinin". Its distribution in human sera has been described by Lind & McArthur[43] It is absent in sera from infants and tends to be increased in sera showing cold agglutinins The nature of T-agglutinin is unknown The possibility that it is a nonspecific agent acting in virtue of the diminished electronegative charge of virus on RDE-treated cells needs to be considered. It has recently been shown that such cells are also agglutinated by purified urinary mucoprotein and by congo-red (Burnet[12]) There is evidence that new antigenic groups are produced or uncovered in RDE-treated cells If a rabbit is immunized with RDE-treated human cells and the serum absorbed completely with normal human cells, a residual capacity to agglutinate the treated cells remains (Burnet & Anderson[11])

Action of heat on influenza-virus haemagglutinin

Francis[30] showed that when the influenza B strain Lee was heated to 56°C for 30 minutes its haemagglutinin titre was unaltered When, however, such heated virus was used to titrate sera for their capacity to inhibit haemagglutination, it was found that *normal* serum showed a high inhibitory titre Analysis of this phenomenon showed that the inhibition of heated Lee virus was due to serum mucoproteins, and that a variety of mucoproteins other than those in serum were equally effective Purified or semipurified preparations from egg-white (ovomucin (Gottschalk & Lind[35])), urinary mucoprotein (Tamm & Horsfall,[57] Ada & Gottschalk,[2] Burnet[12]), meconium (French, Curtain & Pye[32]), and human sputum (Curtain, Marmion & Pye[25]) have been studied, while crude preparations of a variety of glandular mucins and some ovarian-cyst contents (French, Gunter & Motteram[33]) are also active McCrea[46] has also prepared highly active material from human red-cell stromata and sheep salivary glands The active inhibitor in all these materials is enzymically destroyed both by active unheated Lee virus and by RDE. Heated Lee virus has no enzymic activity, and all experiments have shown a complete negative correlation between enzymic activity and what we have called the indicator

state, i.e., virus in which the haemagglutinin is inhibited to high titre by mucoprotein inhibitors.

As would be expected, indicator virus does not spontaneously elute from red cells to which it is adsorbed. If agglutination takes place at room temperature it can be reversed and the cells stabilized by treatment with dilute anti-Lee serum. In the case of chicken cells, agglutination by heated Lee virus at 37°C results in firm irreversible union (Burnet [11]). The cells can be stabilized by treatment with RDE to remove unoccupied receptors, but the existence of firmly attached virus can then be shown by the agglutinability of the cells by anti-Lee immune serum. This phenomenon of firm union by indicator virus Lee is also shown by indicator virus Mel, but not by any active virus, nor with human cells.

Except for two unusual variants, all influenza A and B viruses can be converted to the indicator state either by simple heating or by heat in the presence of a calcium de-ionizing agent at an alkaline reaction (Stone;[52] Edney & Burnet [26]) The negative correlation between enzymic activity and the indicator state holds throughout, but the change-over now tends to be less clear-cut with such a strain as Mel than with Lee (Edney & Burnet [26]).

The nature of the change to indicator has been extensively discussed (Burnet,[8, 9] Smith [49]) and although it cannot be claimed that the interpretation is universally acceptable, it is still the author's contention that *one must assume an inactivation of the capacity of the enzyme groups to* split the substrate, with retention of their specific adsorptive relation to the substrate. In the case of the firm union shown by heated Lee virus, this must be regarded as an alternative result of the secondary phase of mutually oriented interaction.

Interaction of mumps and Newcastle-disease viruses with red cells

Mumps and ND viruses affect the same receptors as the influenza viruses A and B but show some characteristic differences, the most notable of which is their capacity to haemolyse cells. Much more work has been done with ND virus (NDV) but, as far as it goes, experience with mumps has given essentially similar results. In the present connexion, the behaviour of these two viruses is relevant only in its bearing on the interpretation of the results with influenza virus, and discussion will be confined to a brief account of the reactions of NDV.

Most strains of NDV show rapid stabilization of initially agglutinated cells, but these cells can readily be shown to carry firmly-attached enzymically active virus When washed free of unattached virus they will produce agglutination of fresh cells—agglutination which, on standing at 37°C, will itself disperse Treated cells are agglutinable by anti-NDV immune serum and, in the case of the Victorian strain, by *high dilutions*

of serum from a majority of patients with infectious mononucleosis (Burnet & Anderson [15]). In our experience (limited to the Victorian strain), this firm union takes place only at temperatures above 30°C. At room temperature the virus behaves in all respects like a typical influenza virus.

Haemolysis with untreated allantoic-fluid virus is variable and incomplete. If virus is partly purified by methanol precipitation in the cold (Burnet & Lind [18]) or, after dialysis, repeatedly frozen and thawed (Henle, personal communication, Liu [44]), it becomes very much more actively haemolytic, particularly for human erythrocytes. The process of haemolysis appears to be essentially similar to that of the establishment of firm union in two important respects: (1) pretreatment of cells with RDE renders them insusceptible equally to haemagglutination, firm adsorption, and haemolysis by active NDV preparations, (2) neither haemolysis nor firm union takes place at below 30°C.

A discussion of the kinetics of haemolysis will be found in a paper by Burnet & Lind [18].

No evidence of haemolysis by any influenza virus has been reported, and the only connexion with influenza virus reactions is in a possible relation with the firm union shown by heated Lee virus, and by active virus against substrate treated with periodate.

The nature of the influenza virus enzyme

The first clue to the nature of the enzyme incorporated in the influenza virus surface was Hirst's [39] finding that red cells treated with periodate were no longer susceptible to agglutination by virus. This suggested strongly that carbohydrate units were intimately concerned. Since then there has been a steady accumulation of evidence that certain carbohydrate groups incorporated in mucoproteins and mucopolysaccharides form the essential substrate grouping S. Therefore, the enzyme may be referred to as a mucinase.

The actual nature of the S grouping is not yet clear. Gottschalk's work [34] renders it likely that it includes an isoglucosamine unit bound to an amino-acid or short-chain peptide. A substance of this type is liberated from ovomucin by the action of virus Mel. Its complete analysis has so far been rendered impossible by its conversion on hydrolysis into humin substances. All the purified substances that have been studied contain glucose, mannose, fucose, and a hexosamine, as well as amino-acids. An interesting confirmation of the mucoprotein nature of the substrate is Whitten's finding that any of the three normally available types of gonadotrophic hormone loses its biological activity after treatment with Lee virus or RDE (Whitten [60]).

In addition to this chemical evidence, the action of virus enzyme on mucoproteins can be shown in several other ways.

(1) When electrophoretically pure substrate is available, urinary-mucoprotein (Tamm & Horsfall;[57] Ada & Gottschalk[2]), meconium-inhibitor (French, Curtain & Pye[31]), or sputum-inhibitor (Curtain, Marmion & Pye[25]) treatment with virus results in a progressive reduction in its negative charge. In the electrophoretor diagram the peak remains single, but is shifted sharply in the direction of diminished mobility. Meconium inhibitor gives a broad diffuse peak; under the action of virus, however, this is shifted as a whole, indicating that the inhibitor population is to some extent physically heterogeneous, but is functionally homogeneous. The change in the soluble inhibitors is essentially equivalent to the diminished EPM shown by a different technique with treated red cells.

(2) Destruction by RDE and active virus of the action of mucoproteins against haemagglutination by indicator viruses has already been referred to. This is the first and simplest way of demonstrating the enzymic action against a soluble substrate (Burnet[6]).

(3) When either cellular or soluble substrate is treated with minimal concentrations of periodate, irreversible combination of either active or indicator virus will occur With red cells, an interesting series of reagents can be obtained by treating cells successively with the appropriate amount of periodate, virus, and RDE. These " PVR " cells (Fazekas de St. Groth[29]) are stable and are agglutinated by the corresponding immune serum; if indicator virus has been used in their treatment, they are also agglutinable by mucoprotein inhibitors. When a mucoprotein is treated with graded amounts of periodate, a level will usually be found where the inhibitory effect against indicator virus is unchanged and a new inhibitory power against haemagglutination by active virus makes its appearance (Burnet[7]) With further increase in the amount of periodate, the whole inhibitory action of the mucoprotein is destroyed

More work needs to be done to define the first effect of periodate on the substrate, but it appears that the action is essentially to allow primary union followed by secondary orientation—which, however, results not in enzymic breakdown of the substrate but in chemical union.

Interaction of Virus with Susceptible Cell Surface

From the point of view of the intrinsic character of the virus, very little further information has been gained from experiments in which the red cell has been replaced by the susceptible cells of the mouse respiratory tract or the allantoic cavity In the ferret- or mouse-lung preparation in vitro, the general sequence of absorption and elution is the same (Hirst;[35] Fazekas de St. Groth[28]) Destruction of receptors by RDE results in transitory insusceptibility of the tissue to infection (Stone[50, 51]).

Perhaps the point of most interest in the present connexion is the change in the enzymic character of the virus associated with the O-D (original-

phase—derivative-phase) change of influenza A viruses. Stone [53] has shown that the O (human pathogenic) virus reacts poorly with an avian inhibitor (ovomucin) but typically with one of human origin (cyst mucoid). When adaptation to the allantoic cavity is complete with acquisition of power to agglutinate chicken cells, the virus reacts normally with ovomucin.

GENETIC ASPECTS OF INFLUENZA VIRUSES

Until recently it was generally believed that genetic studies on viruses and bacteria must be limited to work on the frequency and mutations appearing particularly in relation to transfer to a different environment. With the advent of microbial genetics, it has become apparent that there are possible types of genetic interaction between micro-organisms which are only remotely related to sexual fusion. "Recombination" between bacterial viruses is now well known, and although the work in my own laboratory has not yet been confirmed elsewhere, its results have been sufficiently striking and reproducible to convince us that very similar recombination occurs in cells simultaneously infected with two different influenza A viruses.

The Pure Clone Concept in Virology

It is becoming evident that any adequate understanding of the changes that take place in viruses under various types of manipulation will need the development of a technical and logical approach analogous to the "pure culture" techniques that have been used in bacteriology proper ever since the time of Koch. It is almost, though by no means quite, unheard of to isolate more than one virus species from a natural case of disease, and there has been a general tendency to think of "a virus" as a collective variable entity always passed from one experimental host to another by massive transfer of infectious material, and adapting itself to new hosts in some unspecified, presumably Lamarckian, process. A much more rigid approach will be necessary if an adequate science of virus genetics is to emerge. When a virus infection is transferred from one host to another, we are setting what is essentially a problem in population genetics. We start with a certain population of virus particles composed, presumably, of a number of variant types, and after passage we have a different population, differently composed, and perhaps containing new mutants not represented in the starting population.

Before we can attempt to analyse such situations, we require to know whether a virus population—influenza virus in the present discussion—can be regarded as equivalent to a normal biological population, in the sense that each morphological unit is also a genetic unit and will give rise

to like descendants, except in so far as mutation gives rise to a new self-reproducing type. The phenomenon of phenotypic lag in bacterial mutation has already provoked doubts about the validity of such a view in bacterial population studies, and it is rather likely that in the case of influenza virus we may have a somewhat indeterminate situation. If the units termed filamentous forms are, as seems highly probable, infectious units, they may contain several times as much genetic material as the spherical forms accepted as the normal morphology of the virus. It therefore becomes possible that a single morphological unit may carry two or more different sets of genes.

Experience with influenza virus indicates that if one starts with two standard laboratory strains, differentiable by half a dozen characteristics, and repeatedly re-isolates them by allantoic passage, each will maintain its characteristics unchanged. By using the method of passage at limiting infective dilution (LD) one can establish that a pure clone virus is being used. The method may be exemplified as follows : a certain allantoic-fluid virus is characterized in regard to six qualities as ABCDEF; it is then titrated in chick embryos at dilutions 10^{-8}, 10^{-8}, and 10^{-7}, giving respectively 0/6, 3/6, and 6/6 fluids containing virus. The three positive fluids at 10^{-8} are each tested and shown to have the characters ABCDEF. One of these

allantoic fluid, some mutations have occurred. Except under extraordinary conditions, however, no mutant will have multiplied sufficiently to rival the numbers of the original type. Passage at LD should therefore ensure that a given strain can be maintained in its original form even if mutants capable of growing more actively than the original in the allantoic cavity should arise On the other hand, if massive inocula are transferred there

which survives

re may well be

the same cell, with the production of morphological units carrying more than one gene complex Even LD passage from such a fluid might require several successive applications before consistently uniform fluids were obtained

Even with influenza viruses, this method of isolating pure clones is tedious and subject to the intrinsic weakness just discussed, but it seems to be the minimum requirement for any serious work on the genetics of influenza viruses.

Mutation in Influenza Virus A

The lability of influenza viruses is known to every worker in the subject. As an example of this lability, it may be of interest to trace the origin of two sub-strains of the classical WS virus with which we have worked. WS virus was isolated in January 1933 from an individual whose case was presumed to be typical of the rather severe epidemic prevalent in Britain at that time. The virus, as isolated, was pathogenic for human beings and for ferrets, it was not pathogenic for mice. In the light of later work we can be reasonably certain that it would not have grown in the allantoic cavity of the chick embryo, it would certainly not have produced lesions on the chorio-allantois, and it would probably have agglutinated human but not fowl red-cells. After several passages in ferrets, it was passed intranasally to mice where, after a few passages, it rather suddenly developed the capacity to produce fatal pulmonary consolidation.

The virus in the mouse-adapted form was sent from Hampstead to Melbourne in 1936. There it was passed on the chorio-allantois of the 12-day chick embryo. At first no indubitable lesions were produced, but after 10 passages distinct proliferative foci were observed. These became better developed with further passage, and between the 20th and 30th passages the embryos began to be affected. By the 40th passage the embryos were invariably dead within 60 hours with gross haemorrhagic lesions in brain and muscles. This strain, subsequently maintained by infrequent allantoic passage and storage in dry-ice, is WSE (Burnet & Lush [21]).

Subsequently Stuart-Harris, starting from similar material, confirmed that WSE-type variants could be obtained by chorio-allantoic passage, and then succeeded in transferring infective material from embryo brain o the mouse brain. After merely surviving there for 11 passages, the virus gradually became more virulent, eventually killing mice to high titre, with acute encephalitic symptoms. After 100 such mouse-brain passages, the strain was sent to Melbourne and, when transferred to the allantoic cavity, represents our strain NWS (Stuart-Harris's "Neuro-flu" [34]).

Some of the main differences between these strains are shown in table I. Without specific analysis of the process it is impossible to be dogmatic, but the impression is very strong that each of the major changes in pathogenicity was the result of several mutational steps.

There are many other laboratory examples of variation in influenza viruses (see Burnet [9]), but none has yet been adequately analysed by genetic techniques. Much more important are the changes in serological character, and perhaps in human virulence, that have occurred naturally in influenza A virus since 1933. The discussion of these is, however, outside the province of this contribution.

TABLE I. CHARACTERISTICS OF THREE WS MUTANTS

Mutant	Pathogenicity for man	Pathogenicity for mouse intra-nasal	Pathogenicity for mouse intra-cerebral	Pathogenicity for chick embryo	Growth in allantoic cavity	Heat stability (haemolytic activity) (°C)	Conversion to indicator
Original WS	+	–	–	–	?–	?	?
WSE	–	+	–	+	++	60	+
NWS	–	+	+	+	+	52	–

Recombination between Influenza Virus A Strains

The first evidence that genetic interaction could occur between influenza virus strains was reported by Burnet & Lind.[19, 20] They inoculated mice intracerebrally with mixtures of the neurotropic strain NWS and a non-neurotropic strain of different serological sub-type, Mel being used in most experiments. Under appropriate conditions of dosage, pure clones of virus were obtained which were serologically Mel but were capable of producing typical fatal encephalitis in mice. By the same general method, three other neurotropic strains were obtained which had the other characteristics of strains SW, Oc I, and a heat-stable WS. In a study of a large number of N-Mel forms (Burnet & Edney [15]) there was a general uniformity of character except for the degree of pathogeniciyt on intracerebral inoculation in mice. A number of strains were obtained which had all the in vitro qualities of N-Mel (differing considerably from both NWS and Mel) but had no power to produce symptoms on intracerebral inoculation in mice. From mixed infections of NWS and a recent A strain, Appleby [5] had also obtained a neurotropic strain with the serological character of the recent strain, and suggested the likelihood of recombination.

During the past year more extensive studies by Burnet, Fraser & Lind [17] and Burnet & Perry [23] have provided many more examples of strains which can hardly have arisen by any other means than genetic interaction. The simplest example to discuss is that of double infections of Mel and WSE. These two A strains differ in six easily demonstrated qualities, so that, if Mel is represented by ABCDEF, WSE will be represented as abcdef. The qualities concerned are as follows .

Aa : Serological type WS or Mel, as shown by haemagglutinin inhibitor tests with appropriate immune sera

Bb : Heat stability · Mel haemagglutinin resists 30 minutes' heating at 62°C while WSE haemagglutinin is destroyed.

Cc : Conversion to " indicator state " by simple heating at 56°C for for 30 minutes , when tested with ovomucin or meconium inhibitor, heated

WSE haemagglutinin is strongly inhibited; heated Mel virus is unaffected except by very high concentrations.

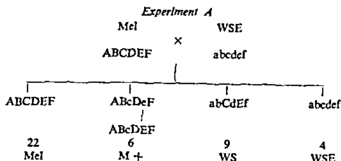
Dd · Reactivity of heated virus with sheep salivary-gland inhibitor (McCrea): WSE is strongly inhibited, no treatment of Mel has ever given a haemagglutinin inhibited by this substance.

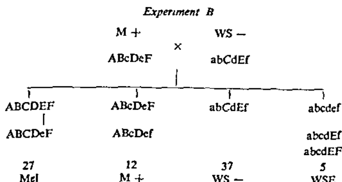
Ee : Production of haemorrhagic lesions in the chick embryo after inoculation on the chorio-allantois : this is characteristic of WSE but is never shown by Mel

Ff : Pathogenicity for mice by intranasal inoculation : with a dose equivalent to one agglutinating unit WSE kills all mice usually four or five days after inoculation; Mel produces non-fatal lesions grading 1-2 on the conventional scale

Double infections are induced in the allantoic cavity by replacing the normal allantoic fluid with a large inoculum of mixed virus, after infection has been initiated, unabsorbed inoculum is removed by washing. As soon as the first-cycle liberation of virus is well under way, the fluid is harvested and its constitution analysed by testing large numbers of LD fluids from repeated titrations The results of two extensive experiments are represented schematically on page 80. In the first, double infection with Mel and WSE gave the two recombinants M+ and WS —. After these had been repeatedly re-isolated, a "back recombination" experiment was carried out with the results shown in the second schema. The distribution of the four in vitro types in the first-cycle fluids is shown by the numbers beneath each group Where there were differences within the group with regard to in vivo characters Ee and Ff, the abnormal types obtained are shown A vertical line from E to e means that strains were obtained with intermediate degrees of lesion-producing power in chick embryos

These results can be taken as establishing that recombination occurs in much the same fashion as with bacterial viruses It is obvious that the situation is complex, and for clearer analysis of the processes concerned it will probably be necessary to study strains differing by single mutational steps The results from both groups of experiments point strongly to the view that development of virulence for a new host is a matter of a sequence of distinct mutations It should be a technical possibility to define fairly clearly how many steps are actually involved, for example, in the adaptation of a virus isolated in the chick embryo to produce active consolidation in the mouse lung From the practical point of view, the important implication of this work is that it is possible to transfer the pathogenicity of a





an effective vaccine strain could be transferred rapidly to any new serological type that might arise in nature.

Morphological units of mixed genetic character

In the course of work on recombination between influenza virus A strains, we have not infrequently found that LD titration of a fluid which gave unequivocal in vitro reactions of a certain type gave fluids of more than one type In each of our papers reference will be found to mixtures being resolved, or difficulty being experienced in obtaining consistent results on repeated LD titration. We have also obtained a number of fluids the haemagglutinin of which was neutralized significantly by *both* antisera. A mixture, of course, will be neutralized by neither. None of these fluids has bred true on LD titration, but that does not invalidate the observation.

Without claiming that no other interpretation is possible, we feel that our results are compatible with the view that morphological units may contain more than one genome and that they are inexplicable if we assume that there is a precise genome-soma relationship in each unit. If, for instance, the average morphological unit carried two discrete genomes, the result of LD titration of the first-cycle fluid from a recombination

experiment in which four products were set free from the mixedly infected cells in equal numbers would probably not give an equal number of fluids showing the four phenotypic characters From the figures given in the section on recombination, and from general experience, it seems likely that, if binary mixtures were inoculated at near-limit dilution, the type appearing as dominant in the final fluid would be that coming first in the series Mel, *WS*−, *M*+, and *WSE* One can calculate that, if the order of dominance of phenotypes in a mixed infection were of this nature, and if free recombination could take place at every mixed infection, then the yield of fluids of each type would be as shown in table II. The results in the Mel × WSE experiment reported would therefore be consistent

TABLE II DISTRIBUTION OF PHENOTYPIC CHARACTERS OF FLUIDS FROM DOUBLE INFECTIONS

Series		Distribution of fluids (%)			
		Mel	WS−	M+	WSE
A	all units with two genomes	56	19	19	6
B	average one unit with one genome to two with two genomes	46	21	21	12
C	average one unit with one genome to one with two genomes	40	22	22	16
D	all units with one genome	25	25	25	25
E	actual findings	56	23	15	10

with there being, on the average, two units each carrying two genomes to one carrying one only (series B)

Any further analysis of the situation will depend on knowledge of the phenotypic expression of compound genotypes In the present exploratory stage of our work all that can be done is to envisage the various possibilities and to hope that appropriate experimental material will be forthcoming to show whether one allele is regularly dominant over the other (if such terminology is legitimate in this type of situation), or whether both types of somatic character are expressed on the unit carrying the two genomes

For the sake of simplicity we have assumed that in all circumstances we are dealing with complete genomes, 1, 2, 3 per morphological unit. A priori, there seems to be no convincing reason why this should be so A virus unit might contain a wholly variable number of genes but be viable only if it contained at least one functioning allele of each of, say, ten specific genes Each phenotypic character might be determined by the predominance of this or that allele of the gene concerned, irrespective of whether the unit were viable or not One interpretation of incomplete virus is that the units concerned lack one or more of the genes needed to give a full com-

plement. The alternative—that they represent somatic material built under genic control but failing to incorporate any genetic material—suggests analogies with turnip-yellows virus but on the whole seems much less likely. On this view, viable virus particles might contain, in addition to the necessary complement of genes, extra genes which might both modify the phenotypic character of the population in which such particles are the dominant form, and allow the emergence of other phenotypes on LD passage

It must be stressed that none of this elaboration of possibilities has as yet any formal experimental background. All that is certain is that there are too many irregularities in recombination experiments to be explicable on the assumption that each particle has its phenotypic and genetic potentialities determined by a single genome of standard pattern.

THE PROCESS OF INFLUENZA VIRUS MULTIPLICATION

In this review I have endeavoured to present the essential facts and the deductions therefrom that bear on what I have called the somatic and genetic aspects of influenza virus. None of this material has any direct bearing on the nature of the process of virus multiplication within the susceptible cell; but, on the other hand, no theory of that process can be acceptable unless it is consistent with the facts in these two categories

The conditions in the allantoic cavity of the chick embryo are sufficiently simple and uniform to make the analysis of influenza virus multiplication in this environment an attractive project. There is now on record a large volume of work from a dozen laboratories, and a fairly consistent picture has emerged. If we take the fully adapted PR8 strain as a model, and where necessary interpolate data from experience with other strains of influenza A or with other susceptible tissues, the essential features of the multiplication process are as follows :

(1) Infection is initiated by adsorption of a virus particle to mucoprotein receptors on the cell surface (Stone;[80, 81] Fazekas de St. Groth[27]).

(2) Once infection has been initiated there is an eclipse period of a few hours during which no viable virus can be extracted from the infected cells.

(3) At some stage before virus is spontaneously liberated into the allantoic fluid, soluble complement-fixing antigen, haemagglutinin, and viable virus can be extracted from disintegrated infected cells There is a majority opinion that the order of appearance of the three agents is as shown.

(4) From the average infected cell approximately 60-120 infective particles are liberated over a period of from two to three hours; 50% of the particles are liberated at approximately eight hours after initiation of infection (Cairns, Fazekas de St Groth & Edney[25]).

(5) Most cells show no sign of serious morphological damage at the time of primary liberation of virus The earliest sign of change is an increase in the pyronine-staining material (ribonucleic acid) in the cytoplasm. Gross damage may or may not follow (Bate, unpublished data)

(6) Infection in cycles after the first is at least predominantly by way of virus liberated into the allantoic fluid

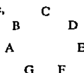
(7) The proportion of incomplete virus produced in the final harvest is a function of the size of the inoculum. No recognizable incomplete virus is produced if the inoculum is less than 10^5 particles (Cairns, Fazekas de St Groth & Edney[28])

(8) The soluble complement-fixing antigen is a particulate agent much smaller (10-15 mμ diameter) than the virus and having a species-specific antigenic pattern distinct from that of any of the virus particle antigens. Its major constituent is nucleoprotein containing ribonucleic acid, and except for the specific antigenic character it cannot be distinguished from the microsomal fraction of normal cells (Ada et al.[3])

The process of virus multiplication in bacteria has been closely studied for some years, and any attempt to construct a picture of influenza virus multiplication in the cell must of necessity draw largely from analogy with bacterial virus.

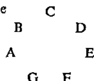
The following attempt to interpret the process of influenza virus multiplication in the cell is almost wholly speculative. Nevertheless, it represents a working hypothesis which offers many suggestions for further experimental work, and may therefore be worth putting on record It obviously owes much to the ideas of Luria, Delbruck, Hoyle,[41, 42] and others.

When the influenza virus enters the cell the soma is discarded and plays no further part The genome in a standard virus particle is a linear aggregate of genes which may be represented ABCDEFG or, as a convention to indicate the completion of the genome,



Mutical configurations are such that C, for instance, will link only with B on the one side and D on the other; similarly, A with B and G; and so on When the genome enters the cell, a process is initiated by which each gene, either singly or in linear fragments—e g, BCD or EF—makes effective contact with cellular units (possibly of microsomal character) which can channel-in energy and material "building stones" for their replication Replicated material will, in turn, be subject to either breakage—e g, BCD may become B, CD—or increase—e g, EF, GA may become EFGA—and these fragments will, in turn, find situations

in which they can replicate. As the pool of genes and gene aggregates comes to contain more and more units, aggregation will dominate over dissociation from simple mass-action considerations We postulate that as soon as a whole new genome



is reconstituted it becomes stable and capable of creating a soma around itself. There are as yet no clues, in either 'phage or influenza virus work, as to how this latter process takes place. When a certain concentration of reconstructed virus particles has been reached, perhaps because of associated damage to the surface cytoplasm, relatively rapid leakage of virus into the environment begins. In terms of the convention being used to represent the complete genome, there is an alternative situation—FGABCDEFGA—which might, perhaps, represent a genetic basis for the filamentous form of the virus and for the apparent existence in recombination experiments of morphological units carrying the potentialities of more than one type of descendant.

Where viruses can produce viable recombinants it must be assumed that their differences correspond to allelic differences in certain genes, so that if one virus is represented ABCDEFG another will be A'BCD'EF'G. It should perhaps be stressed that, as yet, it is not possible to correlate the phenotypic differences used in our recombination experiments with any sort of "chromosome map", nor is there yet any suggestion of how many genes may be needed for a complete virus particle.

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LABORATORY METHODS IN THE STUDY OF INFLUENZA VIRUS

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The aim of the laboratory methods applied to the study of influenza to recognize, identify, and isolate the influenza virus in man, the ly known reservoir, and to reveal the immunological reactions stimulated infected persons Briefly, these techniques serve essentially for the diag- sis of influenza, and for the study of its symptomatology and of the immu- ty produced.

In accordance with a resolution by the Third World Health Assembly,[47, 48] Expert Committee on Influenza was established It met in Geneva on 12 September 1952 and examined, among other questions, laboratory ethods which could be recommended for use in the diagnosis of influenza s conclusions are given in a report [49] which will frequently be quoted in e following discussion *

The problem of diagnosing influenza does not arise solely during idemics and in connexion with influenza-like syndromes Everyday actice has shown that the influenza viruses may be responsible for ymptomatic infections as well as for those showing general symptoms, d pulmonary symptoms which may either be atypical or have an unusual stribution Finally, a diagnosis of influenza is often made by elimina- on at the onset of a febrile affection, or on the basis of pulmonary symp- ms of which the etiology is still obscure

Influenza may be recognized in the patient by various methods :

(1) isolation of the virus, either in the ferret or by culture in the egg;

(2) serological tests in vitro,

(3) cytological tests with animals.

Method (1) affords definite proof of infection It is of particular value t the onset of epidemics, since it supplies the laboratory with strains of n identifiable antigenic type which could if necessary be used for the anufacture of vaccine.

* In particular, the sections on pages 88-89 96-104, 112 116 and 118 119 of this paper are reproduced, ith slight modifications, from the committee's report.

Method (2) provides conclusive proof of infection if it is carried out correctly. According to the technique employed, it offers either information regarding the antigenic type of the causal virus or a simple diagnosis of influenzal infection, recent or still present.

Method (3), when used alone, serves only as an indication. During epidemics, and in a laboratory which is not equipped for serological research, this method may enable a presumptive diagnosis to be arrived at with reasonable accuracy.

COLLECTION AND DISTRIBUTION OF SPECIMENS

In general, the collection and distribution of three sorts of specimen need to be considered. These are, firstly, strains of virus already isolated in a laboratory, secondly, freshly-obtained throat-washings, sputum, etc, for testing for virus; and thirdly, serum samples for diagnostic purposes.

In countries without adequate laboratory facilities, isolation of the virus may often be difficult. This is regrettable, because the information obtained from such virus strains in relation to the spread of the disease cannot be obtained by the examination of human sera. Nevertheless, serological methods utilizing human sera can give useful diagnostic and epidemiological information.

Influenza strains

Strains to be sent to reference laboratories at a distance should be lyophilized if possible. If not, the material should be packed in a container in dry-ice sufficient for the duration of the journey.[b] If dry-ice is not available, ordinary ice may be used in a thermos flask, but only for short journeys (e g, of 36 hours' duration).

Throat-washings and other material

Materials for virus isolation should be sent to a laboratory only if [illegible] glycerol. Throat-washings should be obtained as early as possible in the disease (not later than the fourth day) by getting the patient to gargle with 15 ml of water, physiological saline, bacteriological broth, or skimmed milk. When lavage has been carried out with water or saline, 5 ml of

[illegible]

bacteriological broth should immediately be added to the washings and the whole put as soon as possible in the cold.

Sera

A diagnosis of influenza A, B, or C can usually be made only by examination of two serum specimens from a patient, one taken as early as possible in the disease (and not later than the fourth day), and the other taken 10-14 days after the onset. The titre of influenzal antibodies in human sera is so variable that only by detecting a significant rise (at least fourfold) in titre in the course of illness can the diagnosis be established—hence the need for two specimens At least 2 ml, preferably 5 ml, of each serum should be sent, the serum being removed from the clot before dispatch, whole blood may be sent if the journey to the laboratory occupies less than two days. Care should be taken to avoid contamination during the collection of the blood and the subsequent separation of the serum

Serum samples may be examined by either the complement-fixation test or the haemagglutination-inhibition test. The complement-fixation test is to be preferred for diagnostic purposes in influenza A and B for reasons given on page 97

ISOLATION OF VIRUS

Inoculation in Ferret

Young ferrets, *Putorius (Mustela) furo*, from isolated breeding-stations are used The albino ferret seems to be more sensitive than the polecat ferret, and is the animal of choice for the isolation of influenza virus by animal inoculation (Smith et al.[41]).

The ferret is inoculated intranasally with the pathological material (throat-washings) It is possible to inoculate and infect the animal without anaesthetizing it, but it is nevertheless preferable to use ether anaesthesia, which facilitates handling and aggravates the infection in the inoculated animal

The ferret is placed in a glass jar with a tightly fitting wooden cover. In the bottom of the jar there is a piece of wire netting, bent over at the edges, separating the animal from the bottom A swab soaked in ether is placed in the space between the netting and the bottom The animal is placed in the jar and the lid closed After a period of excitement the ferret becomes quiet and then collapses unconscious on its side It is immediately taken out of the jar and, while still anaesthetized, it is held lying on its back in the left hand, with the muzzle pointing upwards Using the right hand, 5-10 drops of the infective material are dropped into each nostril by means of a fine pipette The animal is allowed to recover consciousness in an isolated cage

After an incubation period of 48 hours, the temperature of the ferret, which normally varies between 38.5°C and 39°C, suddenly rises to above 40 5°C. Subsequently it may fall or show a diphasic form, reaching a peak towards the fifth day. Shortly after the rise in temperature, the ferret becomes drowsy and refuses food; on the third-day it develops catarrh of the eyes and nose : injected, watering eyes, sneezing, nasal obstruction; it breathes with the mouth open; there is a muco-purulent discharge from the nostrils, frequently accompanied by ulceration around the nares. If inoculation has been carried out under anaesthesia, the symptoms are more pronounced; there is some dyspnoea and the animal has a dry cough characteristic of the tracheo-bronchial inflammation. If the infection is allowed to take its course, recovery normally ensues between the tenth and the fifteenth day The animal should be sacrificed preferably when the symptoms reach their height, towards the fifth day after inoculation. The nasal cavities are opened and the swollen, congested turbinates are removed as well as the naso-pharyngeal mucosa. After grinding, this material can be used for inoculation into other animals (ferrets, mice) or, after the addition of antibiotics, for the amniotic inoculation of eggs.

Inoculation in Mouse

The mouse is susceptible to the influenza virus (Andrewes et al.;[1] Francis[10]), but nevertheless it is not suitable for the isolation of virus from infective material from the patient. For transmission of virus to the mouse, the virulent material must be inoculated intranasally under ether anaesthesia, and the strain must first have been adapted to the ferret; as a general rule, recently isolated strains of influenza virus are not pathogenic for the mouse When a recently isolated strain proves to be [illegible] contamina- [illegible] preliminary anaesthesia being essential, even with adapted strains The mice to be inoculated are placed in the jar containing ether vapour and are taken out when unconscious They are then liberally inoculated intranasally (3-4 drops per nostril) and isolated in a jar. With adapted strains this [illegible] The first [illegible] notionless [illegible] Autopsy [illegible]

Inoculation in Hamster

The golden hamster (*Cricetus auratus*) may be substituted for the ferret for isolation of the virus (Taylor & Parodi [45]) The technique to be employed is exactly the same as for the inoculation of the ferret. Nevertheless it must

be realized that, although it may be more economical to use the hamster, this animal is much less susceptible than the ferret to the influenza virus and frequently reacts only by an increase in antibody titre to the standard strain corresponding to the virus inoculated.

Inoculation in Incubated Egg

Inoculation into the egg has become the method of choice for the isolation of virus strains. It allows of rapid adaptation of the virus and its characterization by serological methods. The method is economical, enables more tests to be carried out, and is less liable than animal inoculation to expose the material inoculated to laboratory contamination.

Inoculation for the isolation of virus strains must be performed by the amniotic route (Burnet[4]) Whatever the type of virus concerned, fertile eggs which have undergone preliminary incubation for 8-12 days at 39°C are used In principle, it is preferable to use 12- to 13-day-old eggs with virus A, and 8- to 10-day-old eggs with virus B. In practice, conditions half-way between these limits are employed, and eggs are used on the 11th day of incubation The material to be inoculated is gently centrifuged if it is cloudy, and antibiotics are added (1,000 units of penicillin or 200 μg of streptomycin per millilitre) Inoculation is carried out using one of the following techniques

Intra-amniotic inoculation

We shall not give in detail here the common techniques for inoculation of eggs which have been fully described elsewhere, particularly by Beveridge & Burnet.[3] We shall merely indicate below the method of procedure recommended among the many techniques described in the specialized studies

Direct-vision technique (Taylor & Chialvo's method[41]). Open the air sac over an area 2 cm in diameter Place a drop of alcohol on the shell membrane, and open it. Penetrate the chorio-allantoic membrane with fine, blunt forceps. Through the opening grasp a fold of the amniotic sac with the forceps Inoculate with a fine pipette or by means of a syringe with an 0 3-mm needle. Seal the egg with adhesive tape, and incubate vertically with the blunt end upwards

Semi-direct-vision technique Open the air sac as before. Place a drop of alcohol on the shell membrane Open this and locate the eye of the embryo Insert the needle, mounted on a syringe, in this direction and inject when it is felt that the amniotic cavity has been entered

Technique without direct vision. There are three variations of this technique

(1) Hirst[18] places the egg on the candler in a darkroom and turns it

until the embryonic shadow appears close to the surface. A hole is pierced in the air sac and another at the site of the embryo Through the latter hole a fine needle 2 5 cm long is inserted so as to touch the embryo. 0.2 ml is inoculated.

(2) Enders & Levens[9] work in the darkroom, and locate the embryo as described above. They perforate the shell at the air sac, near the place where the embryo has been located, and push the needle, mounted on a syringe, in the direction of the embryo until it is encountered, when a sudden movement is seen, and a certain resistance is felt. The needle is withdrawn about 1 mm, and the injection is made.

(3) We particularly recommend the method of Sohier & Esser-Trimberger.[42] The eggs are placed vertically in a stand of the egg-cup type in front of a lamp with a point source giving a beam of parallel rays (the type of lamp used for the darkfield microscope). Fit this lamp with a movable screen, either of cardboard, held by a rubber band, or of metal, running backwards and forwards in a groove. The screen should be placed very close to the upper end of the egg so as to mask the light rays. In the majority of cases the various details and reference points will clearly appear. They can be seen better, moreover, by placing the hands one on either side of the egg with the palms turned towards the source of light, or by putting a piece of cardboard with an oval opening in front of the egg The embryo is located and the site of the eye marked with a cross Pierce a hole at the top of the air sac and another at the cross, without touching the shell membrane, and gently insert the needle in this hole. A needle 25 mm long and 0.5 mm in diameter, with a short bevel, should be used; the point may be removed so as to avoid injuring the embryo, but in this case it is more difficult to penetrate into the amniotic cavity. Wait until the movement of the embryo brings it close, and then insert the needle so that the embryo recoils slightly Withdraw the needle a little as soon as contact has been made, and inoculate 0 1-0.2 ml. Seal with adhesive cellophane.

The advantages of this method are as follows : simplicity, very great certainty (the error is about 10%), absence of lesions and soiling, and the possibility of carrying out serial inoculations and of performing alternately amniotic and allantoic inoculations The only disadvantage might be poor visibility of the embryo However, the eggs can be sorted in advance and only those with an easily detectable embryo retained for amniotic inoculation The others should be employed for inoculation by other routes.

Collection of amniotic fluid

The simplest method consists in opening the egg and beginning with the withdrawal of the allantoic fluid The amnion is then grasped with

toothed forceps, and is punctured with the tip of a pair of heated forceps Next, the blunt end of a bulb pipette connected to an aspirator is introduced Alternatively, the egg may be placed horizontally, and the cavity above the dropped chorio-allantoic membrane cut into with scissors and the egg tilted backwards, so that the amnion protrudes This can be recognized by its clear, watery contents; it holds 2-3 ml of liquid, sometimes less. It is then punctured with a mounted needle

Once inoculated, the eggs are left to incubate at a temperature of 35°C and examined daily Eggs of which the embryos die within the first 24 hours after inoculation are rejected The eggs with living embryos are opened 48 hours after inoculation The percentage of embryos dying before 48 hours is variable It may be nil without implying failure in the isolation of the virus The allantoic fluid is collected in sterile haemolysis-tubes stoppered with a cotton or rubber plug A drop of liquid from each sample is placed on a microscope-slide and a little chick-embryo blood diluted in saline is added Mixing is carried out by gently rotating the slide, and the results are read after about 10 minutes at room temperature After the first amniotic passage, agglutination does not generally occur In this case amniotic passages are continued until mixing amniotic fluid and diluted chick-blood produces, within about 10 minutes, a macroscopically visible agglutination of the cells, similar in appearance to a mixture of serum and incompatible red blood-cells The virus may then be adapted to the allantoic cavity, and characterized serologically (see page 94)

The number of blind passages to be carried out from amnion to amnion is variable In exceptional cases, adaptation is obtained on the first passage Usually two or three passages are necessary for the amount of virus to be sufficient If on the fifth passage no agglutination is observed, the attempted isolation must be considered as negative

The adaptation of virus to the allantoic cavity does not involve any particular difficulties Special care must be taken to avoid injuring the blood-vessels when fluid is removed, so as not to impoverish the culture by fixation of the virus on the red blood-corpuscles

The technique employed is simple, only the main points are given below.

Inoculation into allantoic cavity

There is a choice of two techniques

(a) *Injection through air sac* Perforate the shell 1 cm from the air sac The needle used should be of the type suitable for intramuscular injection, 0 7 mm in diameter and 30 mm long, with a short bevel It is inserted to a depth of 12-14 mm, measured from the hole in the shell The quantity injected should be 0 05-0 2 ml

(b) *Direct injection* Using the candler, a pencil-mark is made at a point

until the embryonic shadow appears close to the surface. A hole is pierced in the air sac and another at the site of the embryo. Through the latter hole a fine needle 2.5 cm long is inserted so as to touch the embryo. 0 2 ml is inoculated.

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The advantages of this method are as follows : simplicity, very great certainty (the error is about 10%), absence of lesions and soiling, and the possibility of carrying out serial inoculations and of performing alternately amniotic and allantoic inoculations. The only disadvantage might be poor visibility of the embryo. However, the eggs can be sorted in advance and only those with an easily detectable embryo retained for amniotic inoculation *The others should be employed for inoculation by* other routes.

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(b) *Direct injection* Using the candler, a pencil-mark is made at a point

on one of the large diameters of the egg, 2-3 cm from the base of the air sac, at a site where no blood-vessels are visible. The shell is punctured without damaging the shell membrane. After applying a drop of 10% iodine solution in alcohol, 0 05-0.2 ml of inoculum is injected through the puncture, using a needle 0 5 mm in diameter and 15 mm long, with a short bevel, which is inserted to a depth of 3-4 mm.

Removal of allantoic fluid

Leave the eggs, placed vertically with the pointed end downwards, for a few hours in the refrigerator.

Cut open the shell over the air sac. Carefully remove the shell membrane Draw aside the chorio-allantoic membrane without injuring the blood vessels Aspirate the fluid, pushing aside with a spatula or a small spoon the embryo and membranes which may tend to block the aspirating pipette. The allantoic fluid is light-yellow in colour and sometimes cloudy. Usually 5-7 ml are obtained per egg. A simplified method consists in placing a drop of alcohol on the shell membrane, after opening the air sac, so as to make the vessels clearly visible and then perforating, with the point of a pair of heated forceps, first the shell membrane and then the chorio-allantoic membrane. Introduce the blunt end of a bulb pipette through the opening. Draw off the liquid.

SEROLOGICAL METHODS

Serological methods are of major importance in the diagnosis of influenza. In most cases they are the sole methods employed to reach a diagnosis or to determine the type of virus in question. When the virus has been isolated (see preceding section, page 89), they enable it to be identified.

Principles of Methods Employed

Complement fixation was the first serological method to be applied to the diagnosis of influenza (Smith [40]), suspensions of a virus-infected organ treated by repeated freezing and thawing (Hoyle [21]) were used as antigen. The antigen can be dissociated from the virus particles (Hoyle & Fairbrother [22]) since it is very small and behaves like a soluble antigen (Lennette & Horsfall [25]) which is specific for the type of the virus but not for the variety within the type (Lennette & Horsfall [26]) Nevertheless, there is an antigen fraction which fixes the complement, which is attached to the virus particle (Friedewald [11]), and of which the narrow specificity is comparable to that obtained using the haemagglutination-inhibition tests

Whereas the serum of most normal individuals does not contain antibodies fixing the complement with an influenza antigen (Rickard et al.[36]) after an attack of influenza, the serum from 80%-90% of patients gives a positive fixation test (Eaton & Rickard [8])

When complement-fixation tests are carried out with the soluble antigen, the reactions appear at an earlier stage and disappear more rapidly than in neutralization- and inhibition-tests, to which they do not run parallel; on the other hand, fixation-tests performed using the purified virus as antigen follow the curve of the other tests very closely (Wiener et al [46]).

The inhibition-test was developed as a result of chance observations. On opening the egg to extract the infected fluids, it often happens that the worker damages a vessel in the chorio-allantoic membrane, causing a slight haemorrhage. Hirst,[14] followed by McClelland & Hare,[29] observed that when this accident occurred with eggs inoculated with influenza virus, auto-agglutination of the red cells of the chick embryo took place under the influence of the virus They showed that there was elective adsorption of the influenza virus on the surface of the red cells, and that the degree of agglutination was proportional to the amount of virus in the fluid This phenomenon thus affords a means for estimating by an in vitro method the influenza virus content of any given fluid

McClelland & Hare [29] had the idea of applying red-cell agglutination to the titration of the cultivated virus, and then to the determination of the antibody titre in a patient's serum, by observing the neutralization of the influenza virus by the antibodies.

The work of Hirst [15, 16] helped to show the value of this method and to establish the technique and generalize its use. There are numerous variations of the technique which do not differ fundamentally from one another. The most widely used are those developed in the United States of America by Salk,[37] and in Europe—in particular at the Institut Pasteur (Lépine et al [25])

Whatever the serological method employed, it should not be forgotten that the results can be falsified by many sources of error connected with the sensitivity of the antigen, which reacts to traces of metal in solution, to old distilled water, etc , so that it is as well always to use freshly prepared, twice-distilled water (Sautter & Lépine [39]) and rigorously clean glass-ware

Either the haemagglutination-inhibition test or the complement-fixation test should be used, according to the circumstances The former is the more sensitive and accurate, used alone, it enables the worker to determine the group (A, B, or C) of the virus in question, but its technique is more difficult, and it requires a larger number of antigens and more-specific sera than does the complement-fixation method This latter is a refined method which can be simply and rapidly carried out By revealing an increase in the antibody titre in two samples of serum from the same patient, this test makes it possible to diagnose the presence of an infection of

influenzal origin. It does not allow of the determination with certainty of the antigenic variety of the virus involved. The WHO Expert Committee on Influenza unanimously decided that the complement-fixation method was the method of choice for the clinical diagnosis of influenza [49] When the aim of this diagnosis is the epidemiological study of the virus, or identification of the strain, the preliminary results given by complement-fixation should always be supplemented by the haemagglutination-inhibition technique and by isolation of the virus in question.

A description of the techniques recommended by the committee will be found below.

Methods of Comparing and Typing Strains

The haemagglutination-inhibition test is the method of choice for the comparison and typing of strains. Isaacs, Gledhill & Andrewes [23] have given a description of this test for comparing and typing influenza virus strains which establishes all the details of the technique to be followed A divergence of opinion exists, however, as to the reference sera to be used in the test.[c] There are two classes of sera which can be employed: those that result from infection, e.g., in the ferret, hamster, or mouse inoculated intranasally; and those produced by artificial immunization, e.g, in the guinea-pig, rabbit, or fowl inoculated intraparenterally. It is agreed that the main disadvantages to the use of the ferret are that strict isolation of the infected animal must be preserved and that the yield of serum is relatively small. However, the use of ferret sera has enabled differences which were not detected by the use of rabbit or fowl sera to be discerned between virus strains Nevertheless, some workers prefer to use fowl or rabbit sera because of the ease of handling these animals and the large yield of serum. It is emphasized, however, that all types of reference sera enable the main antigenic groups so far recognized to be readily differentiated, provided that the non-specific inhibitors present in these sera are eliminated.

Procedures which have proved satisfactory in the preparation of ferret, rabbit, and fowl antisera for these purposes and for the elimination of the non-specific inhibitors are given on pages 99-100.

It is important that the typing of strains should be carried out using early egg-passage virus before their characteristics have been altered by artificial cultivation. Freshly isolated strains should therefore be sent to the reference laboratories in London and New York.

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c We use the term " reference serum " deliberately to designate the specific serum, instead of the term

Standard Diagnostic Procedures

Standard procedures are now available for the diagnosis of influenza virus infection, and full details of the techniques recommended for two of these are given on pages 100-116. They are described as a guide for those unfamiliar with the techniques, and are based upon methods which have proved satisfactory in the hands of experienced workers.

From the standpoint of clinical diagnosis, it is necessary to determine only which type of infecting virus is concerned. The so-called soluble antigens (A or B) employed in the complement-fixation test described will distinguish between infections caused by viruses A and B, avoiding difficulties introduced by the existence of variants within the A and B groups. Moreover, the antibody formed against this type of antigen persists for a shorter period than the haemagglutination-inhibiting antibodies. In the case of influenzal pneumonia, in which the virus infection may precede bacterial complications by several days, the titre of antibodies may already have reached a high level at the time of admission to hospital. The results of the test are not affected, as with the haemagglutination-inhibition method, by the presence in the serum of non-specific substances which produce effects resembling those of specific antibody. Thus, in the complement-fixation test using the soluble antigen with serum from a case of influenza, the first or acute sample of serum has either small amounts or no detectable amount of antibody. The convalescent sample will give good complement-fixation.

The haemagglutination-inhibition test is relatively more specific than the complement-fixation test in that, during convalescence, antibodies may appear in the serum which are detected only by using an antigen prepared from related strains of virus. It is therefore essential to employ either more than one type of antigen, or else the actual infecting strain of virus, if that is known. Again, the haemagglutination-inhibition test is affected by non-specific inhibitors present in the sera. The acute sample of the serum will thus give a certain level of inhibition against the virus antigen, unless steps are taken to destroy the inhibitors, as already mentioned in the case of animal sera. Nevertheless, even if inhibitors are not destroyed, the convalescent serum will, by virtue of its enhanced content of antibody, produce a greater degree of inhibition against the appropriate virus.

Preparation of Reference Sera for Comparison and Typing of Strains of Influenza Virus

Preparation of ferret serum

After a preliminary removal, under anaesthesia, of blood from the heart, two ferrets are inoculated intranasally with 1 ml of a 10^{-2} dilution of freshly harvested, infected allantoic fluid. The animals are kept under strict isolation and are bled out 12 days after the original inoculation.

To each serum after separation 1/10,000 merthiolate is added, and the sera are then stored at 2°C. Alternatively, they may be stored at −70°C or lyophilized.

After treatment with cholera filtrate, the pre-infection serum specimen is tested for influenza antibody in order to exclude the possibility of natural infection of the ferret before inoculation. The post-infection serum can be used for strain analysis only if the pre-infection serum is devoid of specific inhibitory activity in the haemagglutination-inhibition test.

Treatment with cholera filtrate prepared as described below (see page 99) is considered necessary by some workers. One volume of serum plus four volumes of crude cholera filtrate are incubated overnight at 37°C; the mixture is then heated for 1 hour at 56°C in order to destroy cholera enzyme activity Other workers (Burnet & Stone [5]) recommend the treatment of ferret antiserum with the receptor-destroying enzyme (RDE) of *Vibrio cholerae*, which is prepared from cholera filtrate by absorption with 5% fowl cells for 1 minute at 2°C followed by elution into normal saline for 30 minutes at 37°C. The eluate is diluted in acetate buffer, pH 6 0, and titrated for its ability to destroy non-specific inhibition in normal ferret serum, using a technique similar to that described below. The eluate is then used with immune ferret serum at the highest effective dilution. This dilution is mixed with serum and the whole is incubated overnight at 37°C, followed by heating at 56°C for 1 hour.

Preparation of rabbit serum

The antigen consists of infected allantoic fluid from embryonated eggs 0.25 ml of a 10^{-4} dilution of high-titre, egg-adapted seed virus is inoculated [illegible]

[illegible]

[illegible]

titre are pooled and tested for bacterial contamination (no antibiotic or other antibacterial material is employed)

Healthy young adult rabbits of either sex are inoculated intraperitoneally three times at weekly intervals with 5-ml quantities of whole, infected allantoic fluid, and are bled one week after the third inoculation.

The crude serum is treated for non-specific inhibitors in the manner described below.

Preparation of fowl serum

The antigen is derived from embryonated eggs, previously incubated for 9 days at 39°C, into the allantoic cavity of which 0 4-ml amounts of the appropriate dilution of seed virus (usually 10^{-4} infected allantoic fluid) are inoculated. After inoculation the eggs are incubated at 35°C for 40-44 hours, and the allantoic fluids are harvested following chilling in the

refrigerator. The haemagglutination titre of the fluid for immunization should be at least 1/320 (initial dilution); titres of 1/640 or 1/1,280 are desirable.

After preliminary bleeding, roosters weighing 5-8 pounds (2-3½ kg) are injected intravenously with 5 ml of the infected allantoic fluid and intraperitoneally with 10 ml For newly isolated strains in which the haemagglutinin titres may be low (1/320) it is best to repeat the injections on the following day.

Ten days after the initial injection, the fowls are bled from the heart. (With large roosters, 80 ml can be removed on three successive days.) The blood is allowed to form a clot, which is cut into small pieces, and is stood overnight in the refrigerator, followed by 5 or 6 hours at room temperature (to aid contraction of clot). The serum is removed, inactivated at 56°C for 35 minutes, lyophilized in 0 5-ml or 1-ml amounts, and stored at +4°C. Storage of the undried serum at −20°C may also be acceptable.

The titres of the pre- and post-immunization sera are determined by the haemagglutination-inhibition test. The immune serum should have a titre of at least 1/800 (initial dilution) with homologous antigen; 1/400 may prove acceptable. The pre-vaccination serum should not inhibit in a dilution of 1/50 Most viruses are not inhibited by normal rooster serum diluted at 1/50, but exceptionally a non-specific inhibition titre of 1/200 may be encountered with newly isolated strains For strain analysis, sera must be treated to remove non-specific inhibitor by incubating for 6 hours at 37°C, with 4 volumes of crude cholera filtrate prepared as indicated below The cholera enzyme activity is finally destroyed by heating at 56°C for 50 minutes, and there is no demonstrable loss of antibody.

Strain analyses are carried out by the standard haemagglutination-inhibition method, using human red-cells. In strain analysis, it is essential that the tests be read after a standard incubation period such as 55 minutes.

Preparation of Crude Cholera Filtrate for Destruction of Inhibitors

Preparation of culture

The strain *V. cholerae* 4Z (Burnet & Stone) is cultivated in nutrient agar containing 2% agar and 2% peptone, pH 7 6, subcultures being made every 3 weeks Subcultures yielding a potent enzyme should be lyophilized as the potency in terms of enzyme production is inconstant and may be decreased by serial subculture.

The seed culture for the preparation of a filtrate is a 6- to 8-hour growth in nutrient broth containing 2% peptone at pH 6 9 From this culture agar plates containing 0 8% agar and 2% peptone (pH 6 9) are inoculated and incubated for 16 hours A heavy growth should be obtained The agar is scraped off the plates, and pressed through sterile gauze (8 layers),

the liquid thereby obtained being subsequently filtered through an asbestos bacterial filter. The filtrate, whose final pH should not exceed 7 6, is stable for long periods at 2°-4°C, but should be tested at frequent intervals for potency as indicated below The quality of the peptone used in the media is a most important factor in preparing a potent filtrate. Different brands of available peptone should be compared, and that found to be most effective should be desiccated and sealed in vacuo. It may then be stored at 4°C.

Potency test

The test for potency of the filtrate is carried out by mixing normal serum with the filtrate in a proportion of 1 of serum to 4 of filtrate and incubating overnight at 37°C, after which the mixtures are heated for 1 hour at 56°C. Sera from ferrets, rabbits, or fowl are all suitable but require separate titrations.

With the treated material, haemagglutination-inhibition tests are set up against two strains of virus The viruses [d] are selected so that one is mouse-adapted and yet retains its sensitivity to inhibitor, and the other is a recently isolated influenza A strain of the FMI group, cultivated only in eggs, which is also highly susceptible to inhibitor.

The actual test is carried out by mixing one volume of test virus con- [illegible] of serum in this mixture is 1/10 At this strength of serum no inhibition of haemagglutination should be obtained with either virus. Dilutions of the serum-filtrate mixture are usually prepared and tested at the same time as an indication of the relative potency of weak filtrates The procedure of mixing virus and serum together half an hour before adding the red cells greatly increases the sensitivity of the test for inhibitor.

Because some batches of cholera filtrate may contain excessive amounts of calcium, which will stabilize the red-cell RDE to heat, it is necessary to include appropriate controls to ensure that the period of heating at 56°C has, in fact, produced inactivation. If not, the batch should be discarded.

Complement-Fixation Test in the Diagnosis of Influenza

The following technique is based upon the procedure recommended by Hoyle,[21] but the antigens are prepared from infected eggs instead of mouse lungs as in the original method.

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[d] *Strains of virus* which have proved satisfactory in the above test are the *mouse-adapted* strain A/Nederland/1941 and the egg-adapted strain A-Barrett/(England)/1947

Reagents

Antigens

The antigens are prepared in one of two ways, both of which have been found satisfactory in different laboratories.

The PR8 or WS strain of influenza A and the Lee strain of influenza B are used in both methods

Method A. Chick embryos are inoculated allantoically after 10 or 11 days' incubation with 0 1-ml amounts of a 10^{-3} or 10^{-4} dilution of infected allantoic fluid After 42-48 hours' further incubation at 35°C, the membranes are removed and suspended in physiological saline (1 ml per membrane), frozen rapidly in a mixture of alcohol and dry-ice, and allowed to thaw out slowly at room temperature. The freezing and thawing are carried out three times, and the suspension is then centrifuged at 3,000 revolutions per minute (r p m) for 15 minutes The supernatant fluid is removed, and 2% chloroform is added to it This is shaken up thoroughly and allowed to stand overnight at 4°C. The suspension is again centrifuged and the supernatant is removed and used as the source of soluble antigen It can be stored at 4°C, but it is recommended that the material should be dried from the frozen state for storage for periods longer than one month.

Method B Nine-day embryonated eggs are inoculated with 0 4-ml amounts of a 10^{-4} dilution of virus-infected allantoic fluid After further incubation at 35°C for 40-44 hours, the eggs are chilled in the refrigerator and the allantoic fluids removed The fluids, which should have titres of from 1/640 to 1/1,280 by the haemagglutination method, are used immediately or stored for an indefinite period in glass-sealed ampoules in the dry-ice box at −70°C

0 5-ml amounts of the undiluted allantoic fluid described above are inoculated in the allantoic cavity of 11-day embryonated eggs After 6 hours' incubation at 35°C, the eggs are opened and the chorio-allantoic membranes are removed These are thoroughly washed in three separate vessels of physiological saline solution, drained on blotting-paper, and weighed They are then ground in a Waring blender for 3 minutes with an equal weight of physiological saline solution to give a 1/2 dilution The material is then centrifuged at 3,000 r p m for 10 minutes to sediment the tissue, and the supernatant is removed Infectivity is destroyed by minimal ultra-violet irradiation, the exact conditions for which will need to be worked out for the individual lamp Tests for infectivity are made in eggs, using antigen both undiluted, and diluted 10^{-1} and 10^{-3}, to obviate interference effects

Normal control-antigens are prepared from uninoculated 11-day embryonated eggs in an identical manner

These preparations are stable for an indefinite period when stored at −20°C or at 4°C.

Sera

Sera are inactivated at 56°C for 30 minutes. If not used at once, either they should be stored at −20°C, or 0 08% sodium azide should be added and the sera kept in the refrigerator (4°C).

Complement

Pooled guinea-pig serum is stored at −70°C. At this temperature the complement maintains its titre over a long period.
In the absence of storage facilities, complement may be preserved by the boric-acid sorbitol azide method of Richardson.[35]

Solution A

Boric acid (H_3BO_3)		1.55 g
Saturated NaCl	to	100 ml

Solution B

Sorbitol ($C_6H_{14}O_6$, ½ H_2O)		9.55 g
Sodium azide (NaN_3)		0 81 g
Saturated NaCl	to	100 ml

Solution C

Sodium azide		0.81 g
Saturated NaCl	to	100 ml

To each 8 ml of serum add 1 ml of solution A and 0 5 ml each of B and C. Store in the refrigerator, and for use dilute 1/8 with distilled water; this gives a 1/10 dilution of complement.

Diluting fluid

Physiological saline is used in connexion with the above antigens, though other types of antigen prepared from eggs may require the use of buffers such as that recommended by Mayer et al.[31]

Haemolytic system

A 2% suspension of packed sheep cells is used, and to this is added an equal volume of haemolysin dilution containing 10 minimal haemolytic doses (MHD)

Titration of complement

A series of tubes is set up containing 0 2 ml of complement dilution (1/10, 1/15, 1/20, 1/25, 1/30, 1/35, 1/40, 1/50, 1/60, 1/70, 1/80, 1/90, 1/100) To each tube are added 0 4 ml of saline and 0.4 ml of sensitized red blood-cells The tubes are incubated in the water-bath at 37°C for 30 minutes A reading is made of the highest dilution giving 100% haemolysis, and also of the highest dilution giving 50% haemolysis (made by throwing down the cells by centrifugation and matching the supernatant fluid against a 1/2 dilution of a tube giving complete haemolysis) For use in complement-fixation tests the complement is diluted so as to contain in unit volume 2½ MHD by the 100% haemolysis reading or 4½ MHD by the 50% haemolysis reading. These two readings usually correspond exactly, when any discrepancy occurs, the 50% haemolysis titre is the more accurate.

Preserved complement retains its titre unchanged for some weeks. After it has once been accurately titrated, it is better to use the same dilution each day than to titrate it afresh every time a test is done In other words, it is better always to use the same amount of complement and to ignore minor variations in sensitivity of the haemolytic system

Titration of antigen

Each batch of liquid antigen prepared is titrated against an excess of a known positive serum. Tubes are set up containing 0 2 ml of antigen dilution (1/1, 1/2, 1/4, 1/8, 1/16, 1/32), 0 2 ml of complement, and 0.2 ml of serum dilution (e g., serum titre 1/64 would be used diluted 1/8). Tubes are incubated for 1 hour in the water-bath at 37°C, 0 4 ml of sensitized cells is added, the tubes are incubated again for 30 minutes, the cells allowed to settle, and readings made Any tube showing less than 50% haemolysis is regarded as positive; 50% haemolysis or more is read as negative

This test gives a reading of the maximal antigen titre The optimal amount of antigen for use in serum titrations is three times the maximal titre, but in practice four times the maximal titre is used, as it is better to have a slight excess of antigen than too little

Standard test for titration of antibody in serum

Each tube in the test contains 0 2 ml of antigen, 0 2 ml of complement, and 0 2 ml of serum dilution.

For normal sera, serum dilutions of 1/2, 1/4, 1/8, 1/16 are used, and for convalescent sera dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256

In addition, a serum control is set up in which a serum dilution of 1/2 is used and the antigen is replaced by saline.

Material and methods

Apart from the preparation of the antigen, which varies with each virus, the same general technique is applicable in practice to all viruses which have been inoculated into animals or embryonated eggs, or obtained by culture in vitro

Antigens

The preparation of influenza antigens is carried out as described on page 101, using influenza virus cultivated in the hen's egg.

The antigens are kept in the liquid state in the refrigerator for use within the normal time-limits.

Reference sera

Every antigen is controlled by using a specific reference serum, which is obtained by inoculation into a suitable animal (ferret, rabbit, or rooster) of a type-strain of the virus under test During the course of immunization, which is generally brought about by repeated injections, tests are made for the appearance of specific antibodies When these have reached the optimum titre the fasting animals are bled out and the blood is left in the refrigerator to coagulate

The serum is then removed, centrifuged, and placed in tubes which are sealed and stored at −20°C. Sera from patients are also centrifuged and stored at −20°C until required for use. Immediately before use, all sera are inactivated by heating in the water-bath at 56°C for 30 minutes

Complement

Batches of sera obtained from healthy guinea-pigs and centrifuged and stored at −70°C are used as complement, freeze-dried complement may also be employed

Diluting fluid

The diluent used as giving the best results is a Veronal (barbital) buffer at with the following formula

NaCl	85 g
5,5-diethylbarbituric acid	5 75 g
sodium 5,5-diethylbarbiturate	3 75 g

Dissolve the acid in 500 ml of hot thrice-distilled water Add the other components and make up to 2,000 ml Then add

$MgCl_2 \cdot 6H_2O$	1 69 g
$CaCl_2$	0 28 g

Autoclave for 20 minutes at 105°C and store at +4°C. Before use, dilute 1/5 in thrice-distilled water.

Red blood-cells

Sheep cells are used, either fresh or preserved in Alsever's solution. Immediately before use they are washed three times in succession in the barbital buffer, then diluted to 1%, and sensitized with an equal quantity of haemolytic serum diluted so as to contain 2 haemolytic units per unit volume and not exceeding this haemolysin content, which is the sensitizing dose facilitating the clumping together of the blood-cells and, consequently, the reading of the result.

Perspex plates

The material used is Plexiglass in sheets, Alsthom M 222 or NP.S 70 (Perspex), 3 mm in thickness. The sheets are cut into rectangular plates measuring 324 × 294 mm. A grid measuring 264 mm along each side and divided into 144 squares, each with a side of 22 mm, is engraved on the plate. The squares are numbered in roman numerals from I to XII down the left vertical edge, each figure corresponding to a horizontal row of squares, and in arabic numerals along the upper horizontal edge, each figure corresponding to a vertical column of squares (see fig. 1, page 108). The plate is fitted with four feet, consisting of 10-mm Plexiglass cubes stuck on at each corner. The grid and the numbers are engraved on the under side of the plate so as to facilitate cleaning of the surface on which the drops are distributed ; this surface should be perfectly smooth and polished.

Pipettes

These are made from finely drawn-out small-calibre glass tubing, as used for Pasteur pipettes. The tapered end is introduced into a calibrated slit exactly 0.8 mm wide, made in a sheet of Dural the size of a playing-card and 1.5 mm thick. This is placed vertically on a suitable support, and a stroke is made with a glass-file across the tapered end of the pipette, flush with the metal sheet , the pipette is then withdrawn from the slit and snapped off sharply It is essential to withdraw the pipette from the slit before snapping it, since without this precaution the line of breakage is not clean and the pipette cannot be used These pipettes deliver about 0 018 ml per drop. One batch of pipettes, tested with the barbital buffer by weighing out 50 drops, gave a margin of error of 0 006 ml per drop, which is in agreement with the tolerance accepted by Kraft & Melnick.[24] Variations caused by differences in the viscosity of the sera are small, and may be considered negligible at the dilutions employed

Air-tight boxes

To avoid evaporation of the drops in the refrigerator or the water-bath, the plates are arranged one above the other in boxes made of galvanized sheet-metal, of which the dimensions are as follows (varying according to whether they are placed in the refrigerator or in the water-bath):

	Refrigerator	*Water-bath*
Length (mm)	370	370
Breadth (mm)	320	320
Depth (mm)	200	120

Each box has a rim 30 mm wide, to which is stuck a band of rubberized material, and upon which rests an overlapping lid, heavy enough to ensure a sufficiently tight fit. Filter paper soaked in water is placed in the bottom of each box to maintain a high degree of humidity. A sheet of filter paper is stuck to the inner surface of the lid with an adhesive, to prevent water of condensation from falling onto the plates

Trays for distribution of the drops

Since the whole reaction takes place in the cold, and distribution of the drops takes a certain time, it is essential to avoid warming-up of the various constituents during the preparation of the tests For this purpose stainless-steel trays are prepared; they are lined with ice-cubes on which a sheet of Dural, measuring 300 × 200 × 5 mm and covered with filter paper, is supported by four stoppers. On this the test plate rests, and is thus maintained during the various operations at a sufficiently low temperature, without, however, its surface misting over and causing the drops to spread All the reagents, which are cooled in advance, are kept in a vessel standing in iced water throughout the course of the operations

Complement fixation

The sera, after being thawed and inactivated, are diluted in tubes with the cold barbital buffer. No serum is used at a dilution below 1/4, any results obtained below this titre being considered as non-specific. Five dilutions ranging from 1/4 to 1/64 are prepared for the sera under test, using 0 2 ml of pure serum. The reference sera for the positive and negative controls are diluted from 1/4 to 1/1,024. All the dilutions are kept in the refrigerator until required for use

The complement, of which the titre for a given batch has been established beforehand, is diluted in tubes so as to contain 2.5 units per drop. The anticomplementary potency of the sera and antigens is determined using the same complement, both undiluted and at dilutions of 1/2, 1/4, and 1/8.

Autoclave for 20 minutes at 105°C and store at +4°C. Before use, dilute 1/5 in thrice-distilled water.

Red blood-cells

Sheep cells are used, either fresh or preserved in Alsever's solution. Immediately before use they are washed three times in succession in the barbital buffer, then diluted to 1%, and sensitized with an equal quantity of haemolytic serum diluted so as to contain 2 haemolytic units per unit volume and not exceeding this haemolysin content, which is the sensitizing dose facilitating the clumping together of the blood-cells and, consequently, the reading of the result.

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Pipettes

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Air-tight boxes

To avoid evaporation of the drops in the refrigerator or the water-bath, the plates are arranged one above the other in boxes made of galvanized sheet-metal, of which the dimensions are as follows (varying according to whether they are placed in the refrigerator or in the water-bath)

	Refrigerator	*Water-bath*
Length (mm)	370	370
Breadth (mm)	320	320
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Each box has a rim 30 mm wide, to which is stuck a band of rubberized material, and upon which rests an overlapping lid, heavy enough to ensure a sufficiently tight fit Filter paper soaked in water is placed in the bottom of each box to maintain a high degree of humidity. A sheet of filter paper is stuck to the inner surface of the lid with an adhesive, to prevent water of condensation from falling onto the plates

Trays for distribution of the drops

Since the whole reaction takes place in the cold, and distribution of the drops takes a certain time, it is essential to avoid warming-up of the various constituents during the preparation of the tests For this purpose stainless-steel trays are prepared; they are lined with ice-cubes on which a sheet of Dural, measuring 300 × 200 × 5 mm and covered with filter paper, is supported by four stoppers. On this the test plate rests, and is thus maintained during the various operations at a sufficiently low temperature, without, however, its surface misting over and causing the drops to spread. All the reagents, which are cooled in advance, are kept in a vessel standing in iced water throughout the course of the operations.

Complement fixation

The sera, after being thawed and inactivated, are diluted in tubes with the cold barbital buffer No serum is used at a dilution below 1/4, any results obtained below this titre being considered as non-specific Five dilutions ranging from 1/4 to 1/64 are prepared for the sera under test, using 0 2 ml of pure serum The reference sera for the positive and negative controls are diluted from 1/4 to 1/1,024 All the dilutions are kept in the refrigerator until required for use

The complement, of which the titre for a given batch has been established beforehand, is diluted in tubes so as to contain 2 5 units per drop The anticomplementary potency of the sera and antigens is determined using the same complement, both undiluted and at dilutions of 1/2, 1/4, and 1/8

The unit of antigen for a given batch is defined as the highest dilution which completely fixes the complement in the presence of the highest dilution of reference serum.

Arrangement of plate

Four different sera are tested on the same plate; it is essential that the same plate carry the different early and late samples from one and the same patient (see fig 1)

The sera are distributed first, this being done vertically on five squares (three for the specific antigens, one for the normal antigen, and one as

FIG. 1. SCHEMA FOR A COMPLEMENT-FIXATION TEST

serum control) A single pipette is used for all dilutions of the same serum, taking care to begin with the highest dilution Thus the first five squares in column 5 receive one drop of 1/64 dilution of serum no 1; the pipette is carefully emptied and then rinsed twice with dilution 1/32, which is distributed in column 4, and so on up to column 1, which receives the 1/4 dilution. Without emptying the pipette, a drop of this dilution is then placed in each of the first four squares in row XI for testing the anti-complementary potency of the serum.

Serum no 2 is distributed in the same way below no. 1, i e., in the first five columns and in rows VI-X The first four squares in row XII are used for anticomplementary-potency control

Sera no. 3 and 4 are distributed one below the other in columns 8,

9, 10, 11, and 12, beginning with column 12 for the 1/64 dilution. Their anticomplementary potencies are checked in squares 5, 6, 7, and 8 of rows XI and XII, respectively.

The barbital diluent is distributed after the sera as follows: one drop in each of the first five squares of column 6 (antigen control which is carried out only once per plate), one drop in rows V and X (serum control repeated each time), one drop in the first eight squares of rows XI and XII (anti-complementary-potency control of the sera), two drops in the last four squares of rows XI and XII (titre of the complement before and after distribution).

Next the antigens are distributed horizontally. Thus, in the case of a test in which three different antigens are employed—a procedure which is unnecessary in practice—one would have:

Type A antigen in rows I and VI
Type A' antigen in rows II and VII
Type B antigen in rows III and VIII
Normal antigen in rows IV and IX
(Rows V and X have already received the barbital buffer.)

The complement is distributed last of all. The rack is taken from the refrigerator at the last moment and kept standing constantly on a tray of ice, a different pipette is used for each complement dilution. As a check on any possible deterioration in the complement during distribution, it is titrated at the outset and at the close of the operation. To do this, one drop of each dilution (i.e., undiluted (2.5 units), 1/2, 1/4, and 1/8) is placed in the last four squares of row XI, the complement containing 2.5 units is then distributed in the horizontal rows I to X and in the first and fifth squares of rows XI and XII, the dilutions 1/2, 1/4, and 1/8 are then delivered respectively into squares 2, 3, 4, 6, 7 and 8 of rows XI and XII, and finally, the last four squares of row XII are used for the final titre of the complement.

The plate is then placed in the air-tight box which is transferred to the refrigerator.

Plate for positive and negative controls

This plate is also used for the control of the anticomplementary potency of the antigens and reference sera. The dilutions of each of the specific sera are distributed in the first three horizontal rows over nine squares.

One drop of the 1/4 dilution of each serum is placed in the first four squares of rows VII, VIII, and IX. A drop of each dilution of the normal serum is deposited in rows IV, V, and VI, over nine squares, beginning always with the highest dilution, the first four squares of row X being used for the anticomplementary-potency controls of the 1/4 dilution. Next, one drop of the barbital diluent is placed in each of the first eight squares

of rows VII-X (anticomplementary-potency control of the sera and antigens) and two drops are placed in each of the first eight squares of row XI (complement controls).

The antigens are distributed horizontally :

Type A in rows I and IV and squares 5–8 of row VII
Type A′ in rows II and V and squares 5–8 of row IX
Type B in rows III and VI and squares 5–8 of row X

Finally, the complement is distributed as with the test plates, beginning and ending with the controls.

After distribution has been carried out on all the plates and they have been piled one on top of the other in the first air-tight box in the refrigerator, a plate carrying no drops is placed on top of the pile. The aims of this precaution are : (*a*) to prevent water of condensation from falling from the lid onto the test plates; (*b*) to make the conditions of evaporation and temperature as similar as possible for all the plates

The container is left overnight in the refrigerator at +4°C; the plates are taken out on the following day and two drops of sensitized red-cells are added to each square. After this the plates are placed in the second air-tight container in a water-bath at 37°C. The result is read after 45 minutes, the plates being examined by transmitted light The degree of haemolysis is noted, ranging from 4 (no haemolysis) to 0 (complete haemolysis).

Schema for, and interpretation of, a complement-fixation test

Fig 1 shows the schema for carrying out a complement-fixation test on a plastic plate. The operation, covering four different sera, is carried out with four different antigens : three specific, and one normal. Thick lines divide the plate into four large squares surmounting three rectangles Each of the large squares corresponds to the tests carried out with the different antigens for a given serum The arrangement and the reagents are the same in each of these squares, which differ only in the serum tested For example, in the first square on the upper left-hand side :

Row I, squares 1–5, first serum with antigen 1
Row II, squares 1–5, first serum with antigen 2
Row III, squares 1–5, first serum with antigen 3
Row IV, squares 1–5, first serum with normal antigen
Row V, squares 1–5, controls (patient's serum and reagents without antigen)

The arrangement is the same in the large square on the lower left-hand side for a second serum, and in the large squares on the upper and lower right-hand sides.

When the sera are arranged in this way, if the first and second sera, or the third and fourth, are the early and late sera, respectively, from the same patient, it is easy to see, by reading the corresponding vertical columns,

whether or not there has been a variation in the complement-fixation titre between the first and second samplings of the patient's serum

In the lower rectangles, from left to right .

Row XI, squares 1–4, anticomplementary potency of the first serum
Row XII, squares 1–4, anticomplementary potency of the second serum
Row XI, squares 5–8, anticomplementary potency of the third serum
Row XII, squares 5–8, anticomplementary potency of the fourth serum

Last rectangle on the lower right-hand side, top line

Row XI, squares 9–12, titre of the complement before distribution
Row XII, squares 9–12, titre of the complement after distribution

Finally, column 6 covers the test for the anticomplementary potency of the different antigens used in rows I-V, as well as in the corresponding rows in the other large squares

Assuming that the test has been correctly carried out, the antigens are shown not to be anticomplementary (column 6) and the complement has retained the same value from the beginning to the end of distribution (lower right-hand rectangle), the test may be interpreted as follows

The first serum (large square on the upper left-hand side) fixes the complement in the presence of antigen 1

The second serum (large square on the lower left-hand side) is negative.

The third serum (large square on the upper right-hand side) is entirely complementary and unsuitable for use

The fourth serum (large square on the bottom right-hand side) is anti-pronounced complement-fixation in the presence of antigen 1, as well as a low degree of fixation in the presence of antigen 3

SPECIAL PRECAUTIONS

(1) *Cleaning of plate* This should be carried out with particular care, since any trace of impurity seriously interferes with both the distribution of the drops and the subsequent clumping of the erythrocytes After careful cleaning with a solution of Teepol the plates are rinsed in tap-water, and then immersed in an alcohol/hydrochloric-acid mixture (45 ml of 90° alcohol , 1 ml of commercial HCl , H_2O q s p 100 ml), and gently rubbed with a cotton-wool pad After having been soaked in this solution for about 10 minutes, they are thoroughly rinsed with tap-water From this time onwards it is important not to rub the plates, for they easily become electrified, making the distribution of the drops impossible since the latter are then drawn from the pipette by electrostatic attraction before reaching their normal size The plates are wiped between two clean cloths, dried with compressed air and then stored between two sheets of filter paper away from dust until required for use

(2) *Distribution of drops* Care should be taken to ensure that the line of breakage of the pipette is sharp and clean , the pipette is held vertically and the distance through which the drop falls should be 1-1 5 cm It sometimes happens—and this seems to be related to atmospheric conditions—that the drops bounce off one another instead of merging In general, this can be overcome by increasing the height from which they fall

to the second tube of the back row. Continue through the series, until each tube of the back row contains 0 25 ml of serial twofold dilutions. Discard the front row of tubes.

3. To each tube add 0.25 ml of saline solution (to bring the volume up to 0.5 ml).

4. Add 0 5 ml of the chicken erythrocyte suspension to all tubes.

5. Include an erythrocyte control of 0 5 ml of saline solution and 0.5 ml of the erythrocyte suspension.

6. Mix well by shaking.

7. Incubate at 22°-24°C, after 60 minutes, read the results on the basis of the pattern in the Kahn tubes (In hot countries where room temperature is over 24°C the alternative of a refrigeration technique (0°-4°C) should be permitted, results to be read after 2 hours, immediately after removal from the cold.)

The 0 25-ml quantity of the highest dilution of the working dilution which completely agglutinates the standard erythrocyte suspension is one unit (i.e , contains one unit of haemagglutinating activity).

Test antigen. The test antigen should contain four haemagglutinating units of virus. This is provided by diluting the working dilution so that it will be four times more concentrated than the highest dilution of the working dilution which showed complete haemagglutination in the above test.

Example : If the greatest dilution of the working dilution which completely agglutinated the erythrocyte suspension was 1/64 (6th twofold dilution), the desired dilution of the working dilution is 64/4, that is, 1 part of working dilution plus 15 parts of saline solution.

The antigen so prepared (test antigen) should be tested in the manner described above (*Haemagglutinating unit*, paragraphs 2, 3, and 4) in order to ensure that it contains 4 haemagglutinating units. Complete agglutination of erythrocytes must be present in the first two tubes of the series, and the third tube must show less than complete haemagglutination.

Standard antisera

Antisera with known inhibitory titre for each of the test antigens should be included in the test

Test for haemagglutination-inhibiting antibody content of sera

Pairs of sera are absolutely necessary for diagnostic tests. The antibody titres of unknown sera are determined by comparing in the same test the pairs of sera, and known antisera, against the standard antigens, in the following manner

(1) All test sera should be inactivated at 56°C for 30 minutes.

(2) For each serum to be tested, set up four rows of Kahn tubes, 10 tubes in each row. Add 1.0 ml of saline solution to each tube of the first row. To the first tube of the first row, add 1.0 ml of a 1/8 dilution of the serum (0.2 ml of serum plus 1.4 ml of saline solution). Mix well, and transfer 1.0 ml of the mixture to the second tube of the first row, using a 1-ml pipette. Mix well, and transfer 1.0 ml of the mixture to the third tube of the first row. Continue through all 10 twofold serial dilutions of this row. With the same pipette, now distribute 0.25 ml of the 10th twofold dilution to each of the last tubes of the other three rows. Then transfer 0.25 ml of the 9th dilution to each of the corresponding (ninth) tubes of the other three rows. Continue with the 8th, 7th, and other dilutions until all have been distributed. The tubes in the front row are discarded and are not used in the final test. If it is desired to use two strains of influenza virus B, it will be necessary to modify accordingly the volumes indicated.

(3) Prepare suitable dilutions of the known antisera.

(4) After the serum dilutions have been prepared, add 0.25-ml amounts of each of the test antigens to each tube of the appropriate row.

(5) To the serum-virus mixtures, add 0.5 ml of the 0.5% erythrocyte suspension.

(6) Shake well and examine to be sure that all cells are well suspended.

(7) Incubate at 22°-24°C for 60 minutes, and then read on the basis of the pattern of the settled cells.

The titre is expressed as the highest initial dilution of serum which complete inhibition of agglutination.

The test antigens must be re-titrated at the same time as the haemagglutination-inhibition tests are performed, in the manner described for the re-titration of the test antigen under *Haemagglutinating unit*, page 113. Again, complete agglutination should occur in the first two tubes of the series of twofold dilutions, and the third tube should show less than complete agglutination.

Titration of virus and antisera may be modified to some extent according to individual preferences. The use of individual pipettes in preparing serial dilutions of antigens or antisera is not necessary, provided adequate care is taken in the use of a single pipette.

Haemagglutination-Inhibition Test in Influenza C Infections

The diagnostic haemagglutination-inhibition test for influenza C has not been entirely satisfactory in all hands, primarily because of the difficulty in preparing antigen in the amniotic cavity. The test has, however, met with success in some laboratories, and a procedure found satisfactory is given below.

Influenza C virus grows poorly in the allantoic cavity of embryo eggs, but the virus can be propagated in high titre in the amnioti The 1233 strain of virus is preferred since it has been found to give h titres than some of the other strains. Embryonated eggs, incubate 10 days at 39°C, are inoculated into the amniotic cavity with 0 1 a 10^{-2} dilution of suitable seed virus preparation After further incub at 35°C for 26-30 hours, the eggs are chilled and the amniotic fluid harvested; the materials are used immediately or stored in 0.5-ml am in lyophilized form

Antiserum to influenza C virus can be prepared in roosters usin basic technique outlined on pages 98-99. However, a single intrave injection of 2.5 ml, and 2.5 ml given intraperitoneally, will usually su Haemagglutination-inhibition titres as high as 1/25,600 have been obta Since all sera tested to date, both human and animal, have appeare

technique described on pages 118-119. Because influenza C virus ra must from red cells at room temperature, titrations of serum and an elutes be made in the cold (4°C) The virus titrations are read after minutes' incubation, and the haemagglutination-inhibition tests after minutes' incubation Human red-cells are employed in the tests

Use of Photometer for Reading Results

With the classic haemagglutination method, whether tubes or pl plates are employed there is no difficulty in recognizing positive haema tination and differentiating it from negative haemagglutination M examination of the bottom of the tubes or plates, particularly if the re are observed in a mirror inclined at 45°, gives a sufficiently character picture to eliminate any doubt in the great majority of cases Neverthe to speed up the reading and at the same time to increase the accurac the response by determining the solution giving 50% haemagglutinat various authors, following Hirst & Pickels [18] and Miller & Stanl employ a method of reading using the photometer The chief import of this technique is that it allows of a serological diagnosis even when increase in the inhibition of the haemagglutination titre between an e and a late serum does not exceed twice the original titre

The technique for taking readings with the photometer – an instrum which is already available in most serological laboratories is given bel

Equipment

Any photometer or densitomet... table for measuring the inten of light transmitted through tu ... ll diameter may be used

light-field to be measured should cover about 1 cm of the length of the tube, measured from a point 5 mm from the bottom of the tube. The tubes recommended are haemolysis tubes with an external diameter of 10-12 mm. A 1.5% suspension of chicken erythrocytes is used. The various operations are carried out in the normal way, the use of an automatic syringe (such as the Becton and Dickinson Cornwall syringe, or its modification devised by Hillemann & Blumberg [13]) is completely satisfactory for this purpose.

Calibration of photometer

The use of a 1.5% red-cell suspension for the test corresponds to a final dilution of 0.75% when the result is read, because of the volume of reagents added. The photometer is consequently adjusted by employing successively an 0.75% erythrocyte suspension, representing the end-point of the titration, and an 0.37% suspension, representing the dilution giving 50% haemagglutination. The sensitivity of the instrument should be adjusted so as to be adequate for concentrations ranging from 0.1% to 0.75% and it is desirable that the 50% point should fall about the middle of the range thus covered. If a photometer which has been adjusted once and for all is employed, it is advantageous before each test to adjust, by dilution, or by the addition of red cells, the suspension of erythrocytes used on the day of the test so as to ensure that its degree of optical absorption coincides with the setting of the instrument.

Titration of virus

The test should be carried out in the classic manner, maintaining the ratio of one volume of virus to one volume of serum and two volumes of red cells, the total volume being 2 ml. The preliminary titration of the virus is carried out by proceeding in the same way with a control dilution, for which normal saline is used instead of serum.

In practice, the virus dilutions are prepared first; twofold dilutions leaving 0.5 ml of virus in each tube are made. Next, 0.5 ml of normal saline is added to each tube, and mixed. In the next stage, 1 ml of 1.5% red-cell dilution is added to each tube. This latter operation, like the preceding one, is greatly facilitated by the use of an automatic pipette. The tubes are then left for 75 minutes in a water-bath at 20°C, or at room temperature if this is close to 20°C. The results are read with the photometer exactly 75 minutes after the final shaking of the tubes. The calculation is readily carried out by finding the two tubes on either side of the 50% point and then taking the photometer readings. The exact 50% dilution of the virus is then interpolated after plotting the two readings taken on a logarithmic scale of dilutions.

Serological inhibition test

The quantity of virus needed to give the 4 agglutinating units per volume required for carrying out the test is calculated from the data prov by the preliminary photometer reading For example, if the titre of virus dilution giving 50% agglutination is 1/57, the antigen shoulc diluted to 1/14.2

After inactivation at 50°C twofold dilutions of the serum under are prepared ; beginning with the 1/8 dilution, 0 5 ml of each dilutio placed in the corresponding tube.

Once the diluted serum has been distributed, the virus, diluted sc to contain 4 agglutinating units per 0.5 ml, is added to each tube and whole is carefully mixed.

Next, 1 ml of the red-cell suspension is placed in each tube, usin suspension freshly prepared from the concentrated stock. Once the conti of the tubes have been mixed, they are left at room temperature for exa 75 minutes. At the end of this period the photometer reading is tal and the two consecutive values of two tubes on either side of the end-pi are noted. The titre of the serum is then interpolated, in the same wa} the haemagglutinating titre

When the method is carefully carried out and the tests repeated w the same virus and the same sera, the error found in the determination the 50% point is less than 10% Under these conditions, an increase the agglutination-inhibition titre of the serum corresponding to twice original titre (i e , a variation of 100%) may be considered significant.

To ensure comparability in different tests made with the same seri a standard serum whose antihaemagglutination titre has been determir once and for all and taken as the reference value, should be emplo} as a control in each series of tests. The same standard serum used dur tests on any given day may, because of the experimental conditions a the sensitivity of the photometer, exhibit differences in reading as compai with the previously established titre The coefficient of variation in 1 reading on a given day is determined with respect to the reference va of the serum, and the same correction is made to the results obtain with the sera examined. The figures thus obtained are of greater val as regards comparability, and enable the tests to be repeated under su ciently accurate conditions

Modification of Haemagglutination-Inhibition Test for Use with Plastic Plates

Titrations of virus and of sera can be carried out on plastic plates inste: of in tubes (Salk [55]). This results in a saving of time in the operation, ar of manpower during the stages of cleaning and preparation of equipmer

Serial twofold dilutions (0 25 ml *) of virus are prepared in physiological saline and equal volumes of 0 5% fowl cells are added Readings are made by the pattern method and the end-point taken as partial (50%) agglutination The 50% end-point is determined by observing the pattern made by a 1/2 dilution of virus showing just complete agglutination. Where partial (50%) agglutination does not show in any dilution, the end-point is determined by interpolation

For titration of serum antibodies, serial dilutions of serum (0 25 ml) are made in saline and an equal volume of red cells is added, followed immediately by the same volume of antigen The antigen is made up to contain 8 partial agglutinating doses of virus per 0 25 ml, and a control test of the concentration of virus actually present is carried out together with the antihaemagglutinin test Partial agglutination is taken as the end-point, and interpolations for the end-point are again made where necessary

Other Serological Methods : Neutralizing Antibodies

It has long been shown that infection with the influenza virus brings about the appearance of neutralizing antibodies in man; these may be detected and titrated according to the classic technique by mixing constant volumes of virus, in concentrations which are definitely infective, with increasing dilutions of the sera under test, using a susceptible experimental animal which may be either the ferret (Smith et al [41]), the mouse (Andrewes et al [2]), or the chick embryo (Hirst [17])

More complicated, more costly, and less rapid in their response than the in vitro methods, neutralization tests are being progressively abandoned in favour of the haemagglutination-inhibition test, the titre and variations of which follow those of the neutralization test so closely that it would seem necessary to accept the conception that the neutralizing antibodies and the inhibiting antibodies are identical in nature (Hirst [15])

THE CYTOLOGICAL METHOD

This method is that described by Panthier, Cateigne & Hannoun [31] It is a purely cytological technique which should preferably be used concurrently with the preceding methods, but which in their absence makes it possible for a laboratory not equipped for serological research to establish a diagnosis on the presumptive evidence resulting from microscopic examination The method depends on the cytological examination of the fluid from the tracheal lavage of mice previously inoculated intra-nasally after anaesthesia, as described earlier (see page 90)

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* The volumes given are those used in conjunction with the plates (manufactured by Prestware Ltd, Lombard Road, London, S W 19 England) as distributed by the World Influenza Centre Plates with different-sized cups will require different volumes

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The quantity of virus needed to give the 4 agglutinating units per unit volume required for carrying out the test is calculated from the data provided by the preliminary photometer reading For example, if the titre of the virus dilution giving 50% agglutination is 1/57, the antigen should be diluted to 1/14.2.

After inactivation at 50°C twofold dilutions of the serum under test are prepared ; beginning with the 1/8 dilution, 0.5 ml of each dilution is placed in the corresponding tube

Once the diluted serum has been distributed, the virus, diluted so as to contain 4 agglutinating units per 0 5 ml, is added to each tube and the whole is carefully mixed.

Next, 1 ml of the red-cell suspension is placed in each tube, using a suspension freshly prepared from the concentrated stock Once the contents of the tubes have been mixed, they are left at room temperature for exactly 75 minutes. At the end of this period the photometer reading is taken, and the two consecutive values of two tubes on either side of the end-point are noted. The titre of the serum is then interpolated, in the same way as the haemagglutinating titre.

When the method is carefully carried out and the tests repeated with the same virus and the same sera, the error found in the determination of the 50% point is less than 10%. Under these conditions, an increase in the agglutination-inhibition titre of the serum corresponding to twice the original titre (i.e., a variation of 100%) may be considered significant.

To ensure comparability in different tests made with the same serum, a standard serum whose antihaemagglutination titre has been determined once and for all and taken as the reference value, should be employed as a control in each series of tests. The same standard serum used during tests on any given day may, because of the experimental conditions and the sensitivity of the photometer, exhibit differences in reading as compared with the previously established titre The coefficient of variation in the reading on a given day is determined with respect to the reference value of the serum, and the same correction is made to the results obtained with the sera examined The figures thus obtained are of greater value as regards comparability, and enable the tests to be repeated under sufficiently accurate conditions

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Starting with the pathological material (throat-washings), several mice are inoculated, and are sacrificed 24, 48, and 72 hours after inoculation For examination, the dead mouse is fixed on its back, and the paws and nose are pinned to a cork mat, care being taken not to stretch the front paws of the animal too much. A lateral incision is made in the skin extending on either side from the front of the lower jaw to the lower part of the abdomen The incisions are flamed with a Bunsen burner and the skin then pulled down. The pretracheal glands are removed, using fine forceps and scissors, and an incision is made in the pretracheal aponeurosis. The trachea, which is readily recognized, has a slightly stretched appearance. A 1-ml paraffin-coated syringe, graduated in twentieths of a millilitre and fitted with a 1-mm needle, is charged with 0 25 ml of normal saline; the needle is inserted in the trachea, which is catheterized as far as possible. The liquid is carefully injected and then withdrawn gently twice in succession; the amount of fluid removed by this process is at least 0 2 ml. It is divided up as follows : one drop is transferred to a slide for microscopic examination after drying and staining, another drop (0.05 ml) is used for titrating the free virus by Hirst's test, and the rest of the fluid is stored after a check for sterility.

The tracheal washings transferred to the slide are dried and then stained, without fixing, by Macchiavello's method [30] Microscopic examination should be carried out using a microscope having a magnification of at least ×1,000. On examination, observations are made of the number and nature of the cells as well as of their internal structure. The washing from a normal mouse shows only a few respiratory-epithelial cells which are broad and not very thick—quite unlike the tracheal washings from an animal reacting to intranasal or tracheal inoculation with influenza virus. In such a case the following appearances may be observed :

(*a*) 24 hours after inoculation with the virus a slight reaction is noted, together with the predominance of normal tracheal cells among which some have a pathological appearance and some are polynuclear.

(*b*) 48 hours after inoculation the appearance is typical : the specimen is covered with an almost continuous unicellular layer. The cells, all of the same type, resulting from the necrosis, followed by desquamation, of the tracheal epithelium, are riddled with round or oval vacuoles regularly arranged in the protoplasm and giving the deformed cell a very abnormal appearance. The protoplasm may be reduced to a mere framework around the vacuoles, and the nucleus, which is displaced to the periphery, is itself often vacuolar and sometimes even dissociated. These characteristic cells are accompanied by polynuclear cells, still relatively few in number.

(*c*) 72 hours after the inoculation, the cell-structure becomes modified. The cells increase in size, and, where previously the vacuoles appeared

to be empty, intensely stained bodies appear at the limit of visibility. The nuclei, which remain strongly affected and displaced to the periphery of the cell, are pitted with vacuoles, and frequently have the appearance of a serrated crescent. The polynuclear cells are very numerous in the smear. Here and there some ruptured cells are noted.

(*d*) After 72 hours the normal tracheal cells reappear, and the influenzal cells gradually disappear. Sometimes a secondary bacterial infection develops, with visible micro-organisms which multiply freely.

The cytological picture returns to normal with the gradual disappearance of the polynuclear cells and the reappearance solely of the normal respiratory epithelium cells.

ELECTRON-MICROSCOPIC EXAMINATION OF VIRUS

An electron microscope is of considerable value in the search for, and direct examination of, the influenza virus. With this instrument it can be seen that the virus is characterized by a very constant morphological appearance—that of a sphere, about 80 mμ in diameter for virus A, and 100 mμ for virus B—which contrasts with the polymorphous aspect of the Newcastle-disease and mumps viruses.

Furthermore, in the case of recently isolated viruses, filamentous orms are found as described by Mosley & Wyckoff[55] and by Chu, Dawson Elford,[6] thus making it easy to recognize a recently isolated strain and distinguish it from a standard or reference strain which has long been tured in the egg.

Direct examination under the electron microscope—preferably after shadowing the virus particles contained in the amniotic fluid, a drop of which is placed on a grid covered by a collodion or Formvar film—may enable the worker to detect a few virus particles. It frequently happens, however, particularly at the beginning of the process of isolation of a strain, that the virus particles present in the liquid are very few in number, necessitating a tedious search. It is quicker, and generally essential, to concentrate the virus before examination.

This concentration may be carried out in two ways, by ultracentrifugation, or by adsorption on red blood-cells. When the liquid is concentrated by ultracentrifugation, the plastic tubes of an ultracentrifuge are filled with amniotic or allantoic fluid as the case may be, taking care to select, when the rotors are interchangeable, a small rotor and small-diameter tubes so as to obtain rapid sedimentation of the virus. Centrifuging is continued for 30 minutes at such a speed that an acceleration of at least 25,000 *g* is applied to the particles. After the centrifuge is stopped, the liquid contained in the tubes is discarded and the sediment is re-suspended in the drop of allantoic or amniotic fluid retained by capillary attraction.

A micro-drop of the re-suspended sediment is transferred to a grid, metallized for shadowing, and examined.

Adsorption on red cells, recommended for the first time by Dawson & Elford,[7] utilizes the property of the influenza virus of adhering to the surface of previously laked erythrocytes, the transparent stroma of which offers little resistance to the electrons. First, 100 ml of 5‰ red cells (chicken or human erythrocytes) are taken, and 5 ml of a pH 6.8-7 buffer and 0.3 g of purified Merck saponin are added. The red cells are lysed in 30 minutes at room temperature. The suspension is then centrifuged for 5 minutes at 4,000 r.p.m., and next washed with buffered normal saline and brought to the original volume. The virus suspension is then added to the laked and washed red cells and the whole left for 30 minutes at room temperature. The erythrocytes are then collected, washed with normal saline, fixed with osmic acid, and transferred to the stage of the electron microscope.

We prefer the following technique to the original one of Dawson & Elford, since it has the advantage that it can be used with a small amount of virus. The suspension of red cells, which have been lysed by the saponin and washed, is transferred to the grid of the microscope previously covered with the collodion or Formvar film The drops of red cells transferred with the pipette are immediately drawn up again, so as to leave only one or two erythrocytes in the field demarcated by each mesh of the grid. The residue from the drop is allowed to dry, and immediately after desiccation of the material a drop of virus, represented, for example, by amniotic fluid, is placed on the grid prepared in this way These operations are carried out in a Petri dish which is then covered and left for 30 minutes at room temperature. When very poor material is used, the drop may even be allowed to dry and a fresh one added After being left for 30 minutes, the drop of virus is drawn up with the micro-pipette, and immediately before drying the specimen is washed by floating on distilled water. It is then dried, shadowed, and examined under the microscope. This very reliable technique has the advantages of making it possible to work with extremely small quantities of amniotic fluid, and of giving a very fine image.

* * *

Recent work by Sampaio & Isaacs (*Brit. J. exp. Path.* 1953, **34**, 152), which has come to the author's attention since this monograph was sent to the printer, has shown that the filtrate of an enzyme with properties analogous to those of trypsin has a destructive action on inhibitors According to these authors, a solution of crystallized trypsin (8 mg per ml in a buffer solution at pH 8, inactivated by heating at 56°C after contact for 30 minutes) can be substituted for *Vibrio cholerae* filtrate. The trypsin

completely destroys the inhibitors without appreciably reducing the specific antibodies The Sampaio & Isaacs technique, which has the great advantage of simplicity, will probably replace the use of crude *Vibrio cholerae* filtrate.

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it did occur in a season in which influenza B was subsequently shown to have been very prevalent. Nevertheless, certain serological tests suggested that some cases of influenza A were occurring. The incidence of afebrile disease was uninfluenced.

The following year (see Stokes, McGuinness, Langner & Shaw[58]) an expanded study in similar institutions was carried out with culture virus of the PR8 strain. In this instance some strains of influenza virus A were recovered from patients with influenza and a reduction in febrile illness indicated that vaccination with active virus had been influential in reducing the incidence of influenza in children, and it was suggested that some of the differences in effect observed in the various institutions might be related to the length of interval between inoculation and appearance of disease

Inactivated virus

In the same winter Smith, Andrewes & Stuart-Harris[55] attempted a controlled prophylactic study, among military forces in England, of subcutaneous vaccination with filtrates of a 10% suspension of the WS strain from mouse lung, inactivated by 1 : 2,000 formalin. The disease occurred before vaccination was fully carried out, and the incidence was low. No effect of the vaccination was noted. A polyvalent vaccine similarly prepared was employed in 1938-9, but no evidence of protection was observed in the group under study (Stuart-Harris and co-workers[59, 60]). Taylor & Dreguss[61] observed no significant effect in their study of vaccinated and unvaccinated individuals, and attention was drawn to the fact that the epidemic strain differed from the WS strain of the vaccine.

The next extensive studies were those of Horsfall, Lennette, Rickard & Hirst[30] with material prepared from chick-embryo tissue previously inoculated with PR8 strain of A virus and a strain of canine distemper virus. The minced embryo suspension was inactivated with 1 : 4,400 formaldehyde, and 1 0-ml doses were given subcutaneously to individuals in a number of institutions. From 30% to 60% of the populations, totalling some 16,000, were vaccinated, the others served as controls. Although a difference in total incidence between vaccinated and controls was observed during an epidemic period, there was a significant reduction in only two of ten vaccinated groups, in two other groups the incidence was higher among the vaccinated than among the controls The ineffectiveness was attributed, in part, to the lack of potency of one large batch of the vaccine. Brown et al.,[1] using a similar preparation, reported an inconstant but final reduction in incidence from 25% in the controls to 13% in the vaccinated groups Dalldorf, Whitney & Ruskin,[2] in a limited study of the same material, noted no difference Siegel et al[52] employed different vaccine preparations through three successive outbreaks of influenza A in 1937, 1939, and 1941, but observed no difference between vaccinated and unvaccinated groups

Conclusions

At this stage, then, there was little consistent evidence from field trials that subcutaneous vaccination afforded effective protection against influenza in times of epidemic, even though studies had adequately shown that vaccination with various materials could induce an increase in antibody titre comparable to that observed after infection. The persistence of satisfactory levels of antibody for a period of several months was the rule, and continued emphasis was placed upon an apparent correlation between the height of the level of antibodies and resistance to the disease. The only conclusions which could be drawn, therefore, were that if influenza was not prevented in the vaccinated individuals it was because (*a*) the vaccine was not of sufficient potency to excite antibody levels to uniformly adequate heights, (*b*) the strains in the vaccine were not sufficiently similar to those causing the epidemic, or (*c*) the level of antibodies in the blood, resulting from vaccination, was not sufficiently high to control influenza.

There were obvious reasons for suggesting that the materials employed in the vaccination studies were not particularly potent in virus content. Eaton & Martin [4] noted that the titres obtained with the complex influenza-distemper vaccine were not as high as those in convalescent patients.

Quantitative determinations of antibody levels, in an attempt to appraise the nature of clinical illness occurring in the study groups, had not been followed extensively until the investigations of Rickard, Horsfall, Hirst & Lennette [40]. These were difficult to interpret because a large number of individuals with high titres who showed no increase in convalescence were classified as having a different disease, influenza Y. This conclusion was dependent to some extent upon Horsfall & Rickard's [31] conclusion that the serological response of the patient convalescent from influenza A was uniform against all strains of type A. Hence, if no increase was observed the causative agent must be of another type.

Effect of concentration on response to active virus

It had been demonstrated in mice vaccinated intraperitoneally with ctive virus that there was a progressive increase in the active immunity obtained as the amount of virus in the inoculum increased up to the point where maximal infective doses were resisted (Francis [7]). Subsequent to these vaccination studies in man, Hirst, Rickard, Whitman & Horsfall [28] carried out a study comparing the effects of different amounts of active and inactive virus obtained from infected chick embryo and from allantoic fluid. The two most concentrated vaccines were prepared by high-speed centrifugation. Using adequate numbers of human subjects, and measuring their antibody levels by the Hirst antihaemagglutination technique, they found with active influenza virus that the antibody titres

It had been possible, however, to demonstrate that excellent antibody responses to both types of virus had occurred, and that in four months a decline of about one-third from the peak titre had taken place (Salk, Pearson, Brown, Smyth & Francis [49]).

Since it had been found, however, that infection could be induced experimentally without serious risk, it seemed desirable to test the efficacy of vaccination in this way. Henle, Henle & Stokes [23] had reported that a mixture of allantoic fluids containing PR8, WS, and Mel strains of type A virus inactivated by formalin, given four months earlier, or inactivated allantoic fluid containing PR8 alone given two-and-a-half weeks earlier, protected all but one of 44 children against inhalation of a recently isolated strain cultivated in eggs (F-99). Ten out of 28 controls became ill.

On the other hand, our studies of resistance to B influenza virus had shown that four months after being sprayed with this virus in allantoic fluid a majority of the people thus infected had symptoms when sprayed again with the same virus, and one-third had just as severe illness the second time, even though their antibody levels were markedly higher than the original titres. On the other hand, a group of 66 vaccinated subjects was selected and sprayed with a strain of type A virus relatively closely related antigenically, but not identical with that used in the vaccine. Of 36 unvaccinated controls, half developed fever of 100°F (37.8°C) or more and symptoms of influenza, of 28 vaccinated four-and-a-half months before, 32% had similar experience, among the 38 vaccinated two weeks before testing, 6, or 16%, had fevers of 100°F—but none higher—in sharp contrast to the other groups (Francis, Salk, Pearson & Brown [15, 16]).

In a group of 96 tested by inhalation of test B virus (Salk, Pearson, Brown & Francis [47, 48]) 11, or 41%, of 27 unvaccinated individuals became ill; of the 79 vaccinated either four-and-a-half months or four weeks—or at both times—before testing, only 8, or 10%, had fevers of 100°F (37.8°C) and none reached 101°F (38.3°C). These results indicated a stronger effect of subcutaneous vaccination than was observed against influenza A, although the B test may have been somewhat less severe. In comparison with the resistance exhibited four months after actual intranasal infection, the effect of subcutaneous vaccination was much more impressive.

In 1943 a more extensive investigation was made by the Commission on Influenza in a series of army units in colleges throughout the USA, with vaccine prepared by adsorption and elution but incorporating equal parts of PR8 and Weiss strains as the A component and Lee strain as B component. The study comprised six different groups of investigators working in nine different universities, and an effort was made to maintain comparable conditions throughout. In all but one area, alternate men of each unit served as vaccinated and controls, the latter being inoculated with physiological salt solution to which formalin (1:2,000) and phenylmercuric nitrate (1:100,000) had been added; the same lots of vaccine were employed

throughout; the same basic plan of clinical observation and handling, and of etiological studies, was maintained.[5, 8, 20, 26, 34, 41, 46, 62] There were 6,263 vaccinated individuals and 6,211 inoculated controls Most of the vaccination was done in late October and early November, and was soon put to the test by an epidemic of A influenza In the total group an incidence of 2 2% of hospitalized cases was observed among the vaccinated persons and 7.1% in the control group, with a consistent and significant reduction in all but the one study in California, where a number of deviations in the pattern of the study occurred, and where Eaton & Meiklejohn[5] attributed the lack of effect to the occurrence of an antigenically divergent strain of virus. In five of the nine units the incidence in the controls was between three-and-a-half and six times as great as in the vaccinated—an effect which may be considered minimal since the frequency of illness among those who had been neither vaccinated nor inoculated was greater than that among controls of the vaccinated groups, thus suggesting a reduction in risk among the controls through the reduction of susceptibility in the group as a whole, owing to the presence of vaccinated individuals

The results clearly demonstrated that a consistent and pronounced lowering in the incidence of clinical influenza A was attained by subcutaneous vaccination with inactive influenza virus

Since in two locations (Hale & McKee,[20] Hirst, Plummer & Friedewald[26]) the epidemic began at about the time of vaccination, the curves of incidence of disease in vaccinated persons and controls could be followed. In the first week no differences were observed, but after six or seven days the curves diverged sharply as the incidence in the vaccinated group decreased; this indicated that the prophylactic effect of vaccine began at a time when circulating antibodies are ordinarily beginning to rise.

In 1945, by virtue of the uniform vaccination of the entire personnel of the US army, and the occurrence of an epidemic of influenza B, it was possible through the Commission on Influenza to gain information about the effect of the same type of vaccine against that disease. At the University of Michigan there were 1,100 men in the unvaccinated naval unit, and 600 in the army unit, all of whom were vaccinated The units lived under similar conditions and were under the medical supervision of the same personnel [illegible]

with similar circumstances and numbers, there were three cases, or 0 5%, among 550 vaccinated army personnel, and 132, or 12 5%, among 1,050 unvaccinated naval students (Hirst, Vilches, Rogers & Robbins[29])

Although these investigations did not employ alternate controls within the same units, the groups were so similar in all other respects as to make them readily comparable. The difference in incidence in the two groups certainly appears to be the effect of vaccination. Further support for this

TABLE I. RESULTS OF VACCINATION AGAINST INFLUENZA IN SIX COMMUNITIES

Location	Vaccinated			Unvaccinated		
	number	cases found	incidence (%)	number	cases found	incidence (%)
Michigan, Mich. . .	600	7	1.2	1,100	109	9
Yale, Conn	550	3	0.5	1,050	132	12.5
Alabama, Ala. .	30	2	6.7	95	18	18.9
Washington, D.C. .	360	7	1.9	4,280	352	8.2
Glasgow, Scotland	115	2	1.7	105	9	8.6
Woolwich, England	609	31	5.1	622	68	10.9

was observed against influenza B than in the earlier studies against influenza A is in keeping with the results noted with experimental infection after vaccination, and also with the readier immunizing effect of B virus in mice. *In addition*, the fact that all members of the one group of units were vaccinated and all those in the control units were unvaccinated may have helped to enhance differences in the mass resistance of the two groups. This influence was exhibited despite the fact that distinct differences could be demonstrated in the serological character of the epidemic strains from that of the Lee strain in the vaccine.

A small group at the University of Alabama also showed a reduced incidence in the vaccinated individuals (Friedman [19]). Norwood & Sachs [38] observed a sharp reduction in an industrial plant in Washington. Two groups were studied by Dudgeon, Stuart-Harris, Andrewes, Glover & Bradley [3] with vaccine of the same character as that used in the USA. The incidence of influenza B was low, and the inoculations were not undertaken until the outbreak was under way Nevertheless, the results at both Glasgow and Woolwich tended to be in favour of the vaccine.

1946-53

Problem of strain characteristics

After a lapse of three years from the influenza A epidemic of 1943, an outbreak of the disease was anticipated in the winter of 1946-7 At the end of October 1946, a vaccination study was again instituted at the University of Michigan, where 10,328 persons received eluate vaccine containing the same strains as in previous years, 7,615 were unvaccinated.

When influenza occurred during March 1947, no evidence of protective effect was demonstrated. The incidence in vaccinated subjects was 7.19% and in controls 8 09% (Francis, Salk & Quilligan[17]). Although the outbreak did not begin until four months after vaccination, the evidence was clear that the antibody titres of the vaccinated individuals, when measured against the vaccine strains, remained at about the same level as those observed two weeks after vaccination. On the other hand, the titres of the vaccinated persons were no higher than those of the controls when tested against epidemic strains

Further evidence of the inefficacy of the vaccine was the high frequency of the disease observed in vaccinated groups, even though comparable numbers of controls were not available (Sigel et al[53]). Data from the US army as a whole yielded no evidence of efficacy[51] In a controlled study, Fowle & Weightman[6] noted incidences of 7.05% in 1,250 vaccinated individuals, and 7.3% in 794 unvaccinated persons Loosli, Schoenberger & Barnett[32] observed the same incidence, 9.5%, in 790 vaccinated and 1,230 unvaccinated individuals, in a test of three different preparations of vaccine Van Ravenswaay[63] observed 20 2% incidence in 237 vaccinated and 27.8% in 284 unvaccinated persons

The British studies[37] involved a variety of institutional and military groups totalling 20,000 persons. The vaccine was prepared by red blood-cell adsorption and elution with either the Mel or the PR8 strain of A virus The incidence of influenza was low, infection was absent in many of the units, and the actual identification of influenza was lacking in others In two schools a mild reduction of incidence was noted in one, from 22% among controls to 11% in vaccinated individuals, and in the other from 17.3% to 11% Otherwise, no differences were observed.

The absence of prophylactic effect was so clear-cut as to differ sharply from, and enhance the significance of, the results of 1943 and 1945. Studies from numerous laboratories clearly showed the serological difference of the epidemic strains from the PR8 and Weiss type A strains incorporated in the vaccine[17, 32, 39, 53, 54] Antibodies to the epidemic strains were not generally induced by vaccination, or occurred only to low levels, although excellent responses to the vaccine strains were demonstrable. There was no significant difference in mean titres to the 1947 strains among vaccinated and unvaccinated persons in the acute stage of the disease, and the antibody increase observed in convalescence was essentially the same in the two groups (Francis, Salk & Quilligan[17]). Furthermore, many sera from the 1943 epidemic, which showed a marked rise to the PR8 strain, failed to show an antibody increase to the 1947 strains. That the strains were of type A was shown by the fact that the majority of convalescent patients exhibited an antibody rise to PR8 or to other A strains, this was demonstrable by neutralization, haemagglutination-inhibition, or complement-fixation tests. That vaccinated individuals

showed less rise to the PR8 than to 1947 strains after infection is to be expected because of their high post-vaccination titres to that strain

The experience of 1947 clearly established an affirmative answer to one question which had been constantly present. Can strain differences demonstrable serologically be of significance in immunization? In order to meet the antigenic variant, the Commission on Influenza recommended that a representative of the 1947 strains, which were designated A-prime, be incorporated in the subsequent vaccines.

Since that time the studies of the Commission have continued in US military installations, with various preparations of vaccine designed to give further information. The major studies are those conducted each year at Fort Dix, New Jersey, and at Fort Ord, California, both in recruit populations.

In the winter of 1947-8, the first of these studies, by Salk & Suriano[30] at Fort Dix, was concerned with comparing the effect of an "old-formula" vaccine containing PR8, Weiss, and Lee strains, prepared by adsorption and elution, with a vaccine containing PR8, FM1 (1947), and Lee, prepared by Sharples centrifugation. The second vaccine induced much better antibody titres to the A-prime strain while the first, although a year old at the time of use, induced somewhat better responses to the PR8 and Lee strains. There was a slight prevalence of A-prime influenza during the period of observation, and while the bulk of respiratory disease in the population was non-influenzal, a significant reduction in the number of cases was demonstrable in the group receiving vaccine containing the FM1 strain.

In 1948-9 the incidence of influenza was so small as to furnish little information about the effect of vaccination upon the disease. However, serological results obtained with monovalent vaccines in that and in the succeeding year have been reported[36] It was readily demonstrated by these means that in man the FM1 vaccine stimulated antibody rises to the PR8 strain of nearly the same magnitude as to itself, although the reciprocal with PR8 vaccine was lacking, as shown in 1947. These data provided a beginning for further interpretation and understanding of strain differences

The following year, 1949-50, influenza caused by A-prime virus was more prevalent The studies at Fort Ord again tested monovalent vaccines, PR8 (A), FM1 (A-prime), and Lee (B), together with control saline inoculations.[35] Equal numbers of each unit received one of the four preparations.

The incidence in the four training groups is shown in the following tabulation:

Vaccine	*Number vaccinated*	*Number of cases*	*Incidence (%)*
FM1 strain	528	5	0 9
PR8 strain	553	21	3 8
Lee strain	536	23	4 3
Control	534	25	4.7

The identification of cases was based upon complement-fixation and haemagglutination-inhibition tests No difference in the incidence of non-influenzal respiratory disease was noted between the groups Thus, despite the low incidence, careful examination revealed that FM1 vaccine had been effective against the current A-prime strain which exhibited a measurable serological difference from that of the vaccine

In 1950-1, in the same installations, two vaccines and saline control were tested The A vaccine contained equal parts of the PR8, FM1 [illegible]

this year have not been published but, at Fort Dix, Dr. J. E. Salk and Dr. E. Lennette (personal communication) observed a 4 1 difference in favour of the vaccinated individuals, and at Fort Ord similar results were noted.

Information from the present year (1953) with still more potent vaccine containing only A-prime strains is incomplete, but preliminary data again indicate a great advantage for the specifically vaccinated group, although the epidemic strain differs serologically from those in the vaccine

The accumulated evidence with epidemics caused by A-prime strains, then, has been uniform in establishing the fact that influenza vaccine containing strains of that group continues to be effective. It also demonstrates that protection can be obtained by the use of vaccine strains which are not identical with those prevalent This is important with regard to both cross immunity and antigenic composition

In 1951-2, influenza B was prevalent Once more, the data are incomplete, but, where the incidence was sufficient to permit measurement, the influence of vaccine was apparent For example, at a children's institution equal numbers within each cottage received polyvalent A vaccine, B vaccine (Lee, 700 CCA units), or saline [24] Although serological differences between the epidemic strain and that in the vaccine were clearly demonstrable, the incidence in the B-vaccinated group, as shown in the following tabulation, was about one-third that in the other two

Treatment	*Number treated*	*Cases*	*Incidence (%)*
B vaccine	207	15	7 2
A vaccine	218	39	17 9
Saline	212	44	20 8

The outbreak was essentially pure influenza B It is of interest that the children in the B vaccinated group who developed the disease were the youngest children, and their antibody titres had shown a sharp decline from the immediate post-vaccination titres This rapid decline in antibody level in three months has not been commonly observed in other studies; it appears to be a factor of age.

Use of adjuvants

In addition to the problem of strain characteristics, the concentration of virus antigen in vaccine is an important factor. In order to obtain high antibody levels, the amount of virus must be maintained well above the minimal level. In the earlier materials prepared by adsorption and elution the amount was essentially that derived from 10 ml of allantoic fluid. Later, an arbitrary level of 300 CCA units per ml was set, and the antibody responses were less marked. Subsequently, the concentrations have been increased to levels of 700-750 CCA units per ml and good titres have resulted. There is, however, the fact that some strains, especially the A-prime group, are less effective even in these amounts, owing to either an inherent antigenic defect or a lesser stability of the inactivated material.

A number of earlier studies had suggested the possibility of using lipids to enhance the effect but, because of serious accompanying reactions, the materials were not acceptable for human use. Recently, Salk and his associates have conducted extensive investigations of the use of virus first emulsified in Arlacel A (mannide mono-oleate)[a] and then suspended in a light mineral oil, a combination found by Freund and his associates[18] to eliminate the unfavourable local abscess production or extensive encephalomyelitic disturbances encountered with certain other preparations.

The studies have developed in a progressive manner, from observations of the responses in experimental animals receiving various combinations of virus and adjuvant to the testing in man of the efficiency and practicability of selected preparations. It was shown first in mice and monkeys that extremely high titres, in the thousands, of haemagglutination inhibitor or neutralizing antibody (in ovo) could be obtained with mixtures of adjuvant and quantities of virus which, in aqueous form, resulted in titres of approximately 128. In monkeys the titres continued to rise progressively from levels of 256 in one week to a peak of 16,000 or more in eight weeks. After four months some decline in titre was commonly observed, but when moderate amounts of virus are considered the levels remain in the thousands at the end of a year. Moreover, an amount of virus which in the usual vaccine had little antigenic effect could, in conjunction with adjuvant, still elicit high titres (Salk, Laurent & Bailey,[45] Salk & Laurent[44]).

The extended studies in man have been presented in two publications (Salk, Bailey & Laurent,[43] Salk[42]) Basically, they demonstrate effects in man paralleling those observed in monkeys. The titres obtained with adjuvant vaccines greatly exceed those obtained with similar amounts of virus in aqueous vaccine. Antibody response to a polyvalent adjuvant vaccine containing 100 CCA units of each of three virus strains reached peak titres of approximately 2,000 in four months, declined in one year to levels of 512-1,024, and remained at around these levels during an additional

[a] Obtained from the Atlas Powder Co., Wilmington, Del., USA

year of observation. In comparison, persons receiving a preparation of aqueous vaccine containing 133 units of each strain had levels of approximately 256, which, with a slight decline, persisted thereafter at half the six-week level. In general, the maximum response to aqueous preparations is reached in two weeks in adults, and at this time usually significantly exceeds the mean titres obtained with adjuvant material. It has been clearly shown, however, that, in the presence of adjuvant, amounts of virus in the range of 10 CCA units can result in levels of antibody well above those following aqueous vaccine containing ten times as much virus This readily offers opportunity for incorporating a number of strains differing antigenically, whereas the quantities required make this more difficult in polyvalent aqueous preparations In this connexion, the data indicate that, with the abundant responses obtained, there is a greater capacity to overcome differences in antigenic structure of various strains It may be pointed out, too, that the contingency of incorporating a new strain immediately into vaccine may well be met by the relatively little attention which need be given to high titres required for production of aqueous material.

The only untoward reactions observed to date occurred in a group of persons given vaccine prepared with a certain lot of Arlacel A These reactions took the form of cyst-like accumulations which developed at the site of inoculation, in about 1% of the subjects, two to four months later. Although many possibilities were considered, it eventually appeared that the cause was impurities or unnecessary substances in that particular lot of emulsifier These have been removed without altering the efficiency of the material, and later preparations have not exhibited this reactivity. In the meantime, tests have been devised for determining the presence of such harmful materials in experimental animals

The possibility of carcinogenic effect has also been explored, and the resultant data indicate that the materials employed do not possess the characteristics associated with the carcinogenic action of oils Similarly, the risk of sensitization appears to be minimal

The efficacy of adjuvant vaccine in protecting man against influenza has not yet been established, but if antibody levels are the deciding factor the evidence weighs heavily in its favour The cost of its production is also considerably less than of aqueous vaccine As with other prophylactic materials, additional data are needed as to the best and most stable strains for use in stimulating antibodies, and for giving wide coverage, accurate knowledge of ideal proportions, and exploration of other materials which may be of still further advantage as adjuvants would also be of great value.

Vaccination against influenza has been shown to be uniformly effective under a variety of conditions, when vaccines of proper constitution and potency are employed. Although the writer has consistently held that the epidemiological and serological data have indicated the probability that strain differences are important in the recurrences of influenza, he

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INFLUENZAL PNEUMONIA: CAUSATION AND TREATMENT *

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Influenza is still a disease with an impact upon the community which can be measured in terms of mortality, yet, compared with the historic outbreaks of 1889-90 and 1918, recent epidemics have been mild However, even recent epidemics severe enough to affect 5%-10% or more of the population have been accompanied by an appreciable rise in the death-rate, particularly in the elderly and in spite of the general use of chemotherapeutic agents Thus the influenza A epidemic in Holland in 1948-9 caused about 2,200 deaths during an eight-week period, moreover, an occasional outbreak with an alarming mortality has been experienced, such as that in 1951 in the town of Liverpool, which suffered an even greater mortality than in 1918 In these exceptionally virulent recent epidemics, however, almost all the deaths have occurred among those aged 55 or over, whereas as many as 50% of the deaths in 1918 occurred in the age-group 20-40 A steady fall in the death-rate in the younger members of the community affected by influenza has been experienced since 1938, which suggests that chemotherapy may be capable of influencing the course of the complications in these cases

The authors' experience of recent epidemics of influenza has convinced them of the need for awareness of the therapeutic problem which may be posed, and which contrasts with the relative ease of therapy of ordinary primary bacterial pneumonia and bronchopneumonia. Also, it is important that the general practitioner should be aware of the danger signals of severe or fulminating influenzal pneumonia, particularly because so much depends upon the speed with which therapy is begun. The relative infrequency of the fulminant case is often a source of additional difficulty when, as during an epidemic, so many patients may demand attention that daily visits become impracticable. The purpose of this article is to summarize the facts known about the pulmonary complications of influenza, and to suggest therapeutic regimes which should be pursued.

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Bacteriology of Influenzal Pneumonia

Bacteria believed to be concerned in the pneumonic process are found in the sputum in cases of influenzal pneumonia, and all fatal cases of influenza, except possibly some of those in the aged or enfeebled, are attributable to bacterial action. Pneumococci predominate in the sputa of patients who develop pneumonia some days after the onset of influenza. Staphylococci of the ordinary pyogenic variety occur in a lesser percentage of cases, but particularly in cases of pneumonia concurrent in time with the influenza virus infection. In view of the normal relative infrequency of staphylococcal pneumonia, and the fact that pneumococcal infection is the predominant cause of ordinary non-influenzal pneumonia, attention deserves to be drawn to the increase in staphylococcal infection which occurs during an epidemic of influenza. Thus 104 (80%) of 130 cases of pneumonia in Sheffield in non-influenzal periods between 1947 and 1951 yielded pneumococci in the sputum. During these periods *Staph. pyogenes* was found in the sputum in only seven instances. During two periods of prevalence of influenza virus A-prime infection—January to March 1949, and January to March 1951—166 cases of pneumonia yielded 114 instances (68%) of pneumococcal infection. Also, during these influenzal periods 33 patients yielded staphylococci either alone or with pneumococci.

During the 1949 influenza A epidemic in Rotterdam, Bruins Slot[1] observed 37 cases of pneumonia, 17 of which were caused by *Staph. aureus*. Fifteen instances of serologically confirmed influenza virus A-prime infection were found among the latter cases. Similarly, one of us (J.M.) has personally observed 25 cases of staphylococcal pneumonia unassociated with primary septicaemia, 18 of which were superimposed on influenza virus A or B infection. It is the authors' opinion, therefore, that the occurrence of severe staphylococcal pneumonia, characterized pathologically by a fibrinous necrotizing inflammation of the tracheal and bronchial epithelium and purulent bronchopneumonia, is a sign of the existence of influenza virus A or B infection.

Unlike experience in 1918, pneumonia caused by the haemolytic streptococcus is relatively rare at present, and organisms such as *Klebsiella pneumoniae* have not been more prominent at times of influenza epidemics than during normal periods.

The exact role of the influenza virus infection in relation to influenzal pneumonia is difficult to discern. There is no doubt of its occurrence but no one can be sure whether the virus infection is limited to the pharynx or whether, as the authors believe, epithelial lesions occurring in the trachea and bronchi are due to virus rather than bacterial action. It is unlikely, except in cases of severe and fulminant pneumonia, that the influenza

[1] Bruins Slot, W. J. (1950) *Ned. Tijdschr. Geneesk.* 94, 3438

virus plays a role in continuing pulmonary infection, and thus treatment directed towards the bacterial component is usually adequate. It is by no means certain, however, that this conclusion is valid for particularly virulent epidemics such as that of 1918. The potentially pneumotropic property of influenza virus cannot be ignored, and some strains of influenza virus may multiply more readily in the human lung than others. Since the first isolation of the influenza viruses in 1933, however, few variations in pulmonary complications in different epidemics which could be attributed to the virus have been encountered. The complications of influenza B appear to be the same as those of influenza A.

Diagnosis

Clinical methods alone may fail to differentiate many cases of influenzal pneumonia because the picture often resembles that of ordinary bacterial pneumonia. However, as these patients are also those who respond most readily to therapy with, for example, penicillin or the sulfonamide drugs, no harm results from incomplete diagnosis. The same cannot be said for the cases of influenzal staphylococcal pneumonia, or, indeed, for severe instances of any variety of influenzal infection. The problem of recognition of the latter cases is, however, a considerable one, first, because such patients should be treated at as early a stage of the disease as possible, and second, because bacteriological assistance and facilities for radiological examination may not be available in the home. The clinical features which should suggest the possibility of pneumonia in patients with either the symptoms of influenza (headache, shivering, myalgia) or a history of recent recovery from influenza are as follows.

(1) *Age.* The probability of complications increases at ages over 50, and patients of 60 and over require to be watched and repeatedly examined. Nevertheless, some cases of the most fulminant form of staphylococcal pneumonia occur in young and middle-aged adults, so that no age-group can be considered as exempt from this complication.

(2) *Previous history of a staphylococcal infection*, such as a furuncle or skin infection in the patient or other members of the family.

(3) *Previously existing chronic disease*, such as diabetes, bronchiectasis, chronic bronchitis, emphysema, and all forms of heart disease.

(4) *Persistent high fever* on the third or fourth day of the disease.

(5) *Occurrence of dyspnoea, cyanosis, chest pain, and a productive cough.* Any or all of these point to the probability of a chest lesion, but the degree of subjective dyspnoea may be slight compared to the rise in respiration-rate, and chest pain may be central in situation rather than in the usual lateral location of a pleural pain. Sputum may not be raised

at all in the most gravely ill patients, but a purulent, blood-streaked, or frankly bloody sputum are usual in any of the varieties of influenzal pneumonia, so that the patient should always be asked to cough in the presence of the doctor in order to permit inspection of any material which may be expectorated.

(6) *Frank signs of consolidation* may exist, but cases of influenzal pneumonia may exhibit extensive radiological changes although the clinical signs are equivocal or even suggestive only of a diffuse bronchitis. Patches of weak breath-sounds and abundant râles may therefore be of greater significance than the absence of bronchial breathing or of dullness.

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Finally, the sequelae of influenzal pneumonia which include lung abscess, pleural effusion or empyema, various degrees of atelectasis, and even rarely pneumothorax, require careful attention in spite of previous chemotherapy. Every effort should be made to obtain the admission to hospital of patients whose conditions fail to resolve within a reasonable period of time (5-10 days), as the differential diagnosis from chest disease such as pulmonary tuberculosis or bronchial carcinoma requires radiological, and possibly bronchoscopic, investigation.

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therapy with other antibiotics is as yet experimental in nature, and recommendations cannot be made at present. There is no particular advantage to be gained by administering antibiotics by inhalation.

The following regime is suggested for patients in whom a definite clinical diagnosis or a presumptive diagnosis of influenzal pneumonia has been made. After the initial clinical examination, aided when possible by radiological examination, the following steps should be taken:

(1) Obtain specimen of sputum, stain by Gram's method a sample thoroughly washed in physiological saline, and set up cultures on blood-agar. Rapid antibiotic-sensitivity tests on the predominant species in the sputum can be carried out by cultivating the sputum on plates with discs containing different antibiotics. If no sputum is available but the patient presents the aspect of a mild or moderately ill case of pneumonia, treat as for pneumococcal infection. If the patient is severely ill, treat as for staphylococcal infection.

(2) If pneumococci predominate in the sputum, treat with penicillin by intramuscular injection of 50,000-100,000 units of ordinary aqueous penicillin every four hours (daily dosage 300,000-600,000 units). If preferred —but only in mild or moderately ill cases—procaine penicillin may be given as 300,000 units intramuscularly twice daily (600,000 units per day), either alone or with 100,000 units of ordinary sodium penicillin. Treatment should be continued for at least 14 days.

(3) If staphylococci predominate in the sputum and the patient is severely ill, penicillin may be given by intramuscular injection at the rate of 1,000,000 units initially, followed by 500,000 units every four hours. If the patient is desperately ill, 1,000,000 units every two hours may be given for the first 12 hours, followed by a lower rate of dosage. This regime may be modified in staphylococcal infections with a lesser degree of clinical severity, but the daily dosage should still be of the order of 1,000,000-2,000,000 units.

(4) If no bacteriological facilities are available, the clinician should be guided by the response to treatment with penicillin at the standard dosage of 50,000-100,000 units intramuscularly every four hours, but should use the higher scale of dosage for all fulminant cases.

(5) If penicillin-resistant staphylococci are reported in the sputum, or the patient fails to respond within 72 hours of initiation of therapy, then a change should be made to a different regime. Oxytetracycline or aureomycin is preferred by the authors because oral therapy (which may be impracticable in severely ill patients who have difficulty in swallowing) can be supplemented by intravenous therapy with the same agent. The requisite daily dosage is still under study, but 4 g daily by mouth should be given for at least 10 days, with lesser daily dosage if intravenous injections are substituted. Treatment may have to be given for at least three

weeks or more. It seems unlikely that any advantage will be obtained by combining either of these antibiotics with penicillin or streptomycin.

(6) *H. influenzae* infections should be treated with penicillin in high dosage (4,000,000 units daily in adults) or preferably with chloramphenicol, aureomycin, or oxytetracycline at the rate of 0.5 g every six hours.

(7) Infections with *K. pneumoniae* should be treated with streptomycin, either alone or with the addition of sulfadiazine or sulfadimidine. If no response is obtained, chloramphenicol, aureomycin, or oxytetracycline may be tried, but the response cannot be predicted.

(8) *Streptococcus haemolyticus* infections may be severe. Optimal treatment has not been studied, but the authors suggest the same dosage of penicillin as in staphylococcus infections.

Ancillary treatment in addition to antibacterial therapy is obvious. Oxygen is of chief value in patients with abundant bronchial secretion and deep cyanosis. It is unnecessary as a routine measure. Peripheral vascular failure may be helped by the use of cortisone and later of adrenocorticotrophic hormone but antibacterial treatment should also be given, and experience is so far totally inadequate for firm recommendation. The use of drugs such as digitalis is not, in the authors' view, of critical importance unless auricular fibrillation or congestive heart failure co-exist. In the latter instance, and also in patients with abundant bronchial secretion, diuretics of the mersalyl (mercury salicyl-allylamide-o-acetate of sodium) variety may assist.

Prophylaxis

Mass prophylaxis of bacterial complications in influenza is impracticable and probably undesirable, because of the possible encouragement of resistant bacterial species or of superinfections with organisms such as fungi, especially *Candida albicans*. Prophylaxis is more reasonable, however, in patients with influenza who are known carriers (nasal or skin) of staphylococci, or who have had a recent infection with staphylococci, in diabetics, and in patients with chronic respiratory-tract or cardiovascular disease. Thus, patients with chronic bronchitis, bronchiectasis, or possibly chronic nasal sinusitis or otitis media, are candidates for the development of a superimposed bacterial infection of the bronchioli and the lung. It is difficult, at present, to lay down rules for the guidance of those who desire to attempt prophylaxis. Sulfonamide compounds are probably prophylactic against pneumococcal or haemolytic streptococcal infections. It is not these organisms, however, but staphylococci and *H. influenzae* which are most dangerous to the subjects already suggested above. Penicillin might be effective prophylactically against staphylococci but there is always the risk of causing the emergence of penicillin-resistant strains. Peni-

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cillin in normal dosage is prophylactically ineffective against *H. influenzae* infections. Less risk appears to exist in the case of chloramphenicol, aureomycin, and oxytetracycline at a level of 2 g orally a day, and these agents are capable of exerting an action against all the bacteria concerned. If used, however, these agents should be given for a period not in excess of a week, and careful watch should be kept for possibly harmful effects, especially with chloramphenicol in regard to the development of inhibition of the bone-marrow.

Mobilization after Uncomplicated Influenza

The elderly patient convalescing from influenza is in a debilitated and weakened condition. The respiratory-tract epithelium has probably not returned to a completely normal state until three to four weeks after the acute illness. It is therefore desirable to urge caution before return to normal occupation, and avoidance of exposure to inclement weather or overcrowded public places.

THE INFLUENZA PROGRAMME OF WHO *

A M-M PAYNE, M D, M R C P.

Division of Communicable Disease Services, World Health Organization

Influenza recognizes no man-made boundaries, indeed, many of the achievements of man increase the speed and extent of its spread. The appearance of epidemic influenza is viewed with concern in the country initially involved, among neighbouring nations, and indeed in all continents It was natural, therefore, that the nations of the world should call on their own intergovernmental Health Organization to play a co-ordinating role in the struggle against the disease

It may be useful first to examine briefly the reasons for concern at the appearance of epidemic influenza, since it will help to define the objectives of a worldwide plan

The first reason is the memory of the 1918-19 pandemic Many readers will recall the appalling suddenness with which it killed healthy young people, the speed with which it spread, and the futility of all efforts to control it It paralysed whole cities, even whole countries food distribution broke down, and the economic loss was enormous It killed more than 15 million people No one knows whether this disaster will ever occur again, for no one knows the combination of circumstances which brought it about However, assuming that it was caused by a variant of the influenza virus, there is a real basis for anxiety because, within certain limits, the virus shows no stability in nature and, as far as is known, a variation that has occurred once may occur again

The second cause of concern is the highly infectious nature of the disease and the fact that it appears to produce no permanent immunity When influenza is epidemic one tends to think—not, whether one will get it, but when will one get it ? Allied to this is its short incubation period and the speed with which it spreads If smallpox broke out 500 miles away, for example, one would not feel anxious, but with influenza one would quite rightly fear that it might arrive within a short period

The third reason is the effect of influenza on the economics of a country. This is naturally very difficult to measure, however, we have only to look at records such as national insurance claims or records of absenteeism in factories to realize that it may be considerable

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* To be published in Spanish in the *Boletín de la Oficina Sanitaria Panamericana*

Finally, but not least, influenza or its main complication, pneumonia, does *kill*. In Liverpool, for example, in 1951 the weekly death-rate exceeded the highest figures of the 1918 pandemic, *although this time it was mainly* the old who died; in the Netherlands in 1949, 2,200 people died within a short period.

This, then, is the objective of the WHO influenza programme: first, to plan against the possible recurrence of a pandemic; second, to devise control methods to limit the spread and severity of the disease; and lastly, to limit the economic effects of an epidemic. Which of the three is regarded as the most important depends on the point of view. However, in the light of present knowledge they can all only be approached in one way.

Before showing how this is being attempted, it is necessary to touch briefly on some technical questions which are really the roots of the whole problem. These are considered in much more detail by Dr. C. H. Andrewes and Dr. T. Francis, jr.[a] Nevertheless, a summary here will help to explain the way in which the WHO programme was developed.

Three main types of the virus of influenza have so far been discovered; the two most important of them—A and B—comprise several subgroups In the case of virus A these may differ so much as to afford little or no protection, after infection or vaccination, from subsequent infection by a virus of a different subgroup.

This was demonstrated in 1947 (Francis et al [4]) when a vaccine made from a strain of virus A (PR8) which had given good results in the 1943-4 outbreak,[7] failed to give any protection at all. It turned out that the virus causing the 1947 epidemic was of another subgroup (FM1) now often referred to as A-prime (Salk & Suriano [6]) which differs considerably from PR8. This subgroup of virus A was first detected in Australia in 1946 (Cam) In retrospect that was a most important observation, because if we had known then what we know now there would have been time to prepare a vaccine before the 1947 outbreak. Nevertheless, the danger of the sudden appearance of a new strain of virus remains one of the most serious problems.

Apart from the antigenic variation just described, strains of virus may differ considerably in their ability to spread and to kill For example, in 1951 the so-called "Liverpool" strain of A-prime virus caused a lethal outbreak in that town, whereas the so-called "Scandinavian" strain spread widely but caused only mild influenza (Isaacs et al.[5]) Yet these strains were so similar antigenically that they were only distinguished by the use of ferret antisera The strains differed also in their power to stimulate antibody production The latter quality is obviously highly important in selecting strains for incorporation in vaccines.

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[a] See pages 9 and 123

The third technical point is that during an epidemic the virus breeds true, within certain limits; that is to say, an outbreak caused by one strain of virus A is not related to one caused by a different strain, even if it occurs nearby and at about the same time For example, in this year's (1953) epidemic the virus responsible for influenza in southern England was sufficiently different from that found in northern France to make it clear that there was no relation between the two outbreaks, even though the Channel is a trivial obstacle to the influenza virus On the other hand the virus responsible for the epidemic in the United States of America was similar to the British virus However, this does not justify the conclusion that the outbreaks were related, because a closely related strain had previously been detected in both countries in 1951 and may have remained there ever since How the virus maintains itself in the intervals between epidemics is not yet known

The consequences of these facts are

(1) that successful vaccination against influenza depends on knowledge of the virus causing the epidemic,

(2) that continuous vigilance is necessary to detect new and potentially dangerous strains of virus at the earliest possible moment; and

(3) that epidemiological reports can be correctly interpreted only in terms of laboratory studies of the viruses responsible

These are the technical conclusions which must be considered in planning to attain the objectives already set out It will be seen that the essential knowledge required is early information regarding the nature of the virus causing an outbreak, and a careful analysis of its characters, especially its antigenic structure, and that this information must be gathered from as wide a geographical area as possible This was appreciated as long ago as 1941, when the US Armed Forces Commission on Influenza, under the chairmanship of Dr T Francis, jr, set up a network of laboratories for the isolation of influenza virus, with a central reference laboratory known as the Strain Study Center under Dr T P Magill, its function, as the name implies, was to study and compare strains of virus isolated in different places Valuable though the work done by this organization was, its usefulness was inevitably restricted by national boundaries.

On 3 April 1947 at its third session the attention of the Interim Commission of the World Health Organization was drawn to the problems and dangers of epidemic influenza by a proposal of the Representative from the Netherlands that a small committee should be appointed to consider the problems [8] After discussion, the Commission instructed the Executive Secretary to send an observer to the Fourth International Congress on Microbiology, to be held in Copenhagen in July of that year, to obtain from the experts gathered there as complete information as possible on the subject At Copenhagen an informal meeting of 45 interested people was

held at the Rigsdag on 25 July, and after discussion a small committee of nine members from nine countries was chosen to consider how the views expressed could best be put into practice. At the committee's request Dr. C. H Andrewes (United Kingdom) prepared a memorandum embodying the suggestions made, which was placed before the Interim Commission at its fourth session in September 1947.[9]

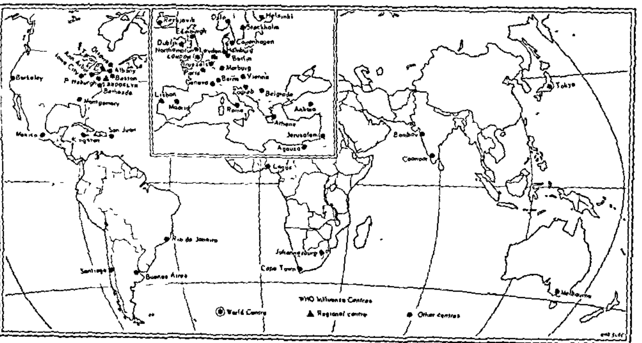
The proposals were that a World Influenza Centre (WIC) should be set up with responsibility for collecting and distributing information, carrying out and co-ordinating laboratory work on influenza, and training laboratory workers. It would work in close co-operation with a number of regional laboratories The Interim Commission [9] accepted the proposals and decided to establish and finance an international influenza centre in England, and to ask Dr Andrewes to begin the work as recommended in his memorandum The Medical Research Council of Great Britain agreed to the establishment of the WIC at the National Institute for Medical Research in London, and the WHO influenza programme had begun.

Work commenced at once, but the organization of a worldwide network of laboratories takes time, and indeed is not completed yet, since in a number of countries there are no virus laboratories and no trained virologists During the winter of 1947-8 the USA was invited and agreed to participate in the co-operative programme (Culbertson; [2] see also Davis [3]) The Strain Study Center under Dr Magill, already mentioned, was designated the National Strain Study Center for the United States of America, and the programme was largely built around it, utilizing the existing facilities for investigating influenza, especially those under the Armed Forces Commission on Influenza. An Influenza Information Center was established at the National Institutes of Health, Bethesda, Md, to administer the programme under a committee designated by the Surgeons-General of the Army, Navy, Public-Health Service, and Air Force, and to act as the liaison office between the WIC in London and the co-operating laboratories in the USA

As the network developed it became clear that there would be great advantages both in speed and in convenience if a single reference laboratory served the whole of the American region. Accordingly the US Strain Study Center accepted designation as the Strain Study Center for the Americas, and now acts for the whole continent exactly as the Centre in London acts for the rest of the world The two reference laboratories co-operate closely so that the overall world picture can be seen.

There is now a total of 54 WHO-designated influenza centres in 42 countries, but of these 27 are in Europe and 11 in North America. In Central and South America there are 6, in the Eastern Mediterranean Region there are 2, in the African Region 3, in South-East Asia 2, and in the Western Pacific 3 A complete list of centres will be found in Annex I (page 161) (see also fig 1)

FIG. 1. WHO INFLUENZA CENTRES



There are other laboratories co-operating informally in various regions, especially in the USA, but it is clear that the network is not yet worldwide. Efforts are being made to extend the coverage with the help of the WHO regional offices (see Annex 3, page 168). As a temporary solution a number of Influenza Observers (see Annex 2, page 167) have been designated. They are unable to undertake laboratory studies but they furnish epidemiological reports.

The functions of an Influenza Centre are twofold :

First, to report with all speed the occurrence of influenza within a country, with an estimate of its extent and severity. This information is sent in parallel to the Epidemiological Information and Morbidity Statistics Section at WHO Headquarters in Geneva, to the appropriate regional office, and to the appropriate reference laboratory in London or New York. In the American Region the arrangement is slightly different. The reports from the USA and Canada are first collected by the national Influenza Information Centers which then transmit the information in the same way.

The collection of epidemiological information regarding influenza presents many difficulties It is well known that notifications are relatively meaningless, because the clinical diagnosis of influenza has no scientific accuracy, and also, during an epidemic the more overworked a practitioner is the less time he has for notifying his cases. In addition, the speed with which influenza spreads makes a delay of only a few days in collecting and distributing the information highly important. What is needed is not a record of actual numbers of cases but some kind of index of the presence of influenza-like disease, based for example on absenteeism among public-transport workers, in factories, or in schools. If such information were collected regularly in selected towns, "normal" figures could soon be established, the simplicity of the information would greatly accelerate the speed of collection, and it might prove possible to follow the trend of an epidemic without the present time-lag. In time of epidemics information regarding the incidence of influenza is collected telegraphically from national health administrations Frequently, however, the first news of an outbreak reaches the WHO Epidemiological Services from an Influenza Centre and is followed by reports of laboratory results. The information is distributed in the several epidemiological weeklies issued and airmailed from Geneva, Alexandria, Singapore, and Washington, and—if sufficiently important—by cable and in the daily epidemiological radio bulletins (see fig 2) In addition, summaries are sent by airmail to all Influenza Centres at regular intervals so that the information regarding the prevalent virus which is needed for the proper use of vaccines is available as soon as possible Of course, if the pandemic recurred much greater use would be made of radio and cabled information.

The second function of an Influenza Centre is to identify the type of influenza by serological tests and preferably by virus isolation The results

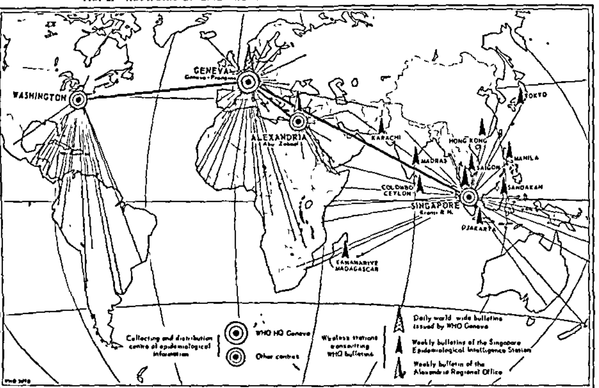


FIG. 2. NETWORK OF EPIDEMIOLOGICAL RADIO-TELEGRAPHIC COMMUNICATIONS

are reported in the same way, and the viruses isolated are dried, frozen, and dispatched by air to the appropriate reference laboratory as soon as possible for further study and comparison with strains isolated elsewhere.

The latter function raises difficulties other than the mere mechanical ones of transport. When an unusual strain is isolated, it is natural to wish to characterize it fully before passing it on to others. This takes time and it is just these unusual strains which are potentially so important. They may be needed at once for the manufacture of vaccine because they may have unusual virulence and ability to spread It is essential for the WHO programme that these strains should be made freely available the moment any unusual characters are recognized. The sense of international responsibility shown by the workers co-operating in this programme has been truly remarkable.

Strains showing obviously unusual characteristics, collected by the two reference laboratories, are exchanged without further delay, so that they are available for vaccine production in both hemispheres if necessary, they are also sent to other Influenza Centres on request. Most strains, however, do not show such unusual features and they are subjected to careful antigenic analysis and characterization by the reference laboratories to clarify their relationship with other strains. In order to avoid changes in the virus which may occur as the result of passage in the laboratory, early egg-passage material should be sent to the reference centres As the epidemic proceeds, the epidemiological and virological evidence accumulates and when it is complete the epidemiological data are interpreted in terms of the laboratory results

It has already been mentioned that it is not known how the influenza virus maintains itself between epidemics, nor is it fully understood how epidemics are generated Sometimes good evidence of geographical spread of the virus is found, sometimes the disease suddenly appears simultaneously all over a large area as if a preliminary seeding of the virus had taken place throughout the population The relative importance of the two methods seems to vary in different areas but it is easy to see the practical significance: if the spread is predominantly geographical (as appears to have been the case in the 1948-9 A outbreak in Europe, which began in Sardinia and spread through Sicily, Italy, and thence through western Europe) (Chu et al [1]) there may be time to vaccinate key-persons before the epidemic wave arrives If, on the other hand, the virus seeds itself almost invisibly and then the epidemic breaks out everywhere at once, it is really too late to do anything which will have much effect It is therefore highly important to understand more about the genesis of epidemics, since the future application of control measures will to a large extent depend on it This investigation is one of the main functions of the two reference laboratories and obviously the study can only be made with international co-operation

Actually a good deal of progress is being made and this is reviewed in the contribution on the epidemiology of influenza by Dr C. H. Andrewes.[b]

In co-ordinating the work of a large number of laboratories in many different countries, it is found that technical procedures vary in different places and sometimes the results obtained are not comparable. The experience of workers varies too, and many virologists ask for advice and guidance on new techniques. It is also important that new knowledge should be disseminated as widely and as quickly as possible, so that its practical application is not delayed. Sometimes special problems arise which need co-ordinated research for their solution. Sometimes several workers, unknown to each other, work for long periods on the same approach to the same problem, causing unnecessary duplication and waste of effort.

To overcome these difficulties WHO has evolved a system of Expert Advisory Panels and Expert Committees. Leading workers in a great variety of fields are invited to serve on these Panels, and undertake to advise on technical matters concerned with their own speciality and to keep WHO informed of important advances. From time to time, as determined by the World Health Assembly, Expert Committees, consisting generally of from six to ten members, are convened to report and advise on specific problems. The reports of the committees, after approval by the Executive Board of WHO, are usually published in the *Technical Report Series*.

An Expert Advisory Panel on Virus Diseases has been established to advise on influenza and other virus diseases, and an Expert Committee on Influenza[c] was convened in 1952. The committee reviewed the work of the WHO programme and made suggestions for more effective international collaboration. It studied certain technical questions, including the methods of comparing and typing strains, and diagnostic procedures, and gave precise details of recommended methods for performing diagnostic complement-fixation and haemagglutination-inhibition tests. It also described the preparation of antisera for the comparison and typing of strains of influenza virus, and the preparation and use of crude cholera filtrate for the destruction of inhibitors. Other subjects briefly reviewed included influenza virus vaccines, the collection and distribution of epidemiological information, control measures, and the therapy of influenzal pneumonia.

The recommendations of the Expert Committee should go a long way towards ensuring comparable results in different laboratories, and this will be aided by the proposed provision by WHO of standard diagnostic reagents to laboratories in the WHO network. A freer exchange of new knowledge

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[b] See page 9

[c] The report of this committee has been published as *World Hlth Org. techn. Rep. Ser.* 1953, 64

can also be hoped for, as well as improved facilities for training workers in the techniques of virology.

It should, perhaps, be emphasized that although the WHO influenza network of laboratories was primarily organized for the study of influenza, very many, if not all, of the laboratories undertake a great deal of work on other virus diseases. The network is therefore potentially able to embark on a co-operative international study of other virus diseases, should such a need arise, and, with that in mind, it is regarded as important that training should cover virology in general and not be confined solely to the techniques used in the study of influenza.

Finally, what has the WHO influenza programme achieved so far, and what of the future ? Space does not permit a full account. Indications as to some of the results have already been given; others are reviewed by Dr. Andrewes, and details can be found in various publications, notably the *Bulletin of the World Health Organization*, to which references will be found in his paper [d]

Possibly the most important achievement is that workers in 42 different countries are working in harmony towards a single end, with no financial reward and sometimes with a partial sacrifice of individual credit for the work. As a result, our knowledge of influenza virus variation and the epidemiology of influenza has increased enormously. The type of virus responsible for an outbreak is now usually known early enough for the information to be of practical value to countries not yet affected. For example, this year it was possible to inform the governments of certain countries which vaccine was the correct one to use before any influenza had broken out there. The choice of strains of virus for inclusion in vaccines clearly requires international consultation. This year, too, when telegraphic news was received of the Japanese epidemic, an unusual virus isolated in Japan in 1951 was at once dispatched by airmail to both Australia and the USA, in case it was responsible, and in time for a limited production of vaccine, had it been necessary. The virus was not responsible, but the mechanism is there and is working satisfactorily.

There are still many unsolved problems in influenza which are thoroughly reviewed by other contributors to this monograph and which need not be repeated here. There is hope that some of them will be solved in the near future, as a result of the extensive research now in progress in many parts of the world

The WHO influenza programme is now a going concern and will continue to grow and improve in efficiency. It is there to apply on an international scale the new knowledge gained by national research, and to supplement that knowledge by international collaboration. Whether influenza will ever be controlled no one can tell, but the time when it

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[d] See page 9

will be possible to limit the effects of epidemic influenza to a significant degree appears to be within sight, and the WHO programme is helping to bring that day nearer.

Annex 1

WHO INFLUENZA CENTRES

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Regional Office of the World Health Organization for the Western Pacific P.O Box 2932 Manila Philippines	UNISANTE, MANILA
Regional Office of the World Health Organization for Africa P O. Box 6 Brazzaville French Equatorial Africa	UNISANTE, BRAZZAVILLE
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9. World Health Organization, Interim Commission (1948) *Off Rec World Hlth Org* 6, 56, 193, 214

ILLUSTRATIONS *

* The illustrations were kindly provided by the Statens Seruminstitut, Copenhagen (fig 1), the World Influenza Centre, London (fig 2 and 3), and the Institut Pasteur, Paris (fig 4-21) Of the latter, fig 4 is from a cliché prepared by the Virus Section for a paper, as yet unpublished by Dr J Vieuchange and Dr J Giuntini, fig 5 and 6 are from clichés prepared by the Influenza Laboratory, and fig 11-21 are reproduced by courtesy of Dr B Fauconnier of this institute

FIG. 1. VIRUS A (DAN/1/50) - ELEMENTARY BODIES AND FILAMENTS

Adsorption onto fowl red-cell ghost



Palladium shadowed

FIG. 2. VIRUS A (ENG/1/51) - ELEMENTARY BODIES AND FILAMENTS
Adsorption onto fowl red-cell ghost

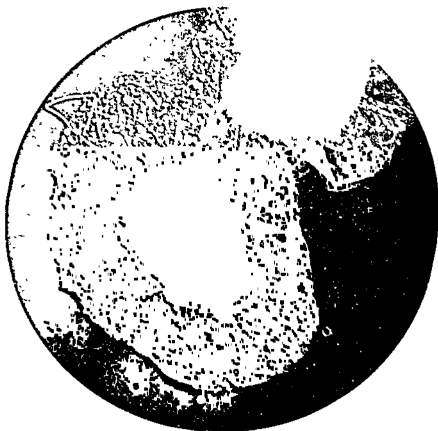


FIG. 3. VIRUS B (LEE) - ELEMENTARY BODIES
Adsorption onto fowl red-cell ghost

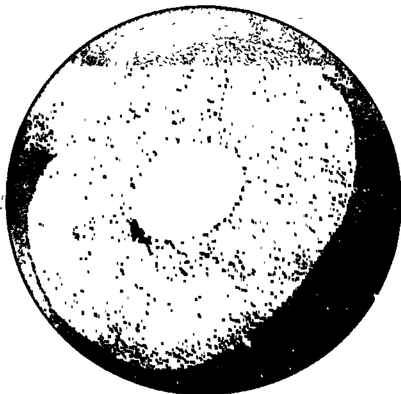
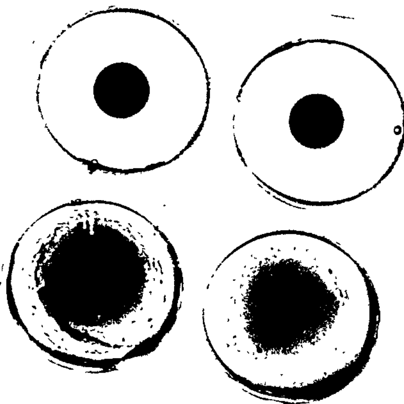


FIG. 4. VIRUS A (PR8) ADSORBED ONTO FOWL RED-CELL GHOST



FIG. 5 HAEMAGGLUTINATION BY SPECIFIC SERA



A — Negative, natural sedimentation of red cells in the centre of the bottom of the tube

B — Positive, agglutination of red cells covering the bottom of the tube

As seen in a mirror inclined at 45°

FIG. 6. HAEMAGGLUTINATION BY SPECIFIC SERA

Series of reactions on a plastic plate

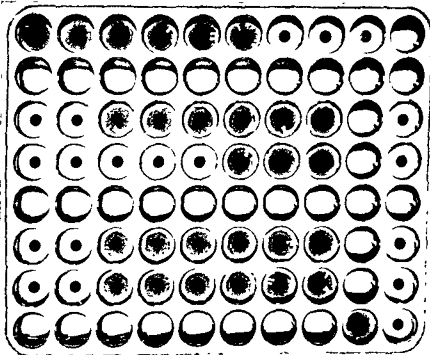


FIG. 7 TRACHEAL WASHING FROM MOUSE — I
24 hours after Inoculation - normal cells



FIG 8 TRACHEAL WASHING FROM MOUSE — II
48 hours after Inoculation - Infected cells

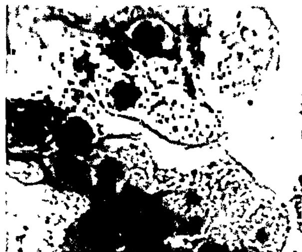


FIG. 9. TRACHEAL WASHING FROM MOUSE — III
72 hours after inoculation - Intracellular bodies

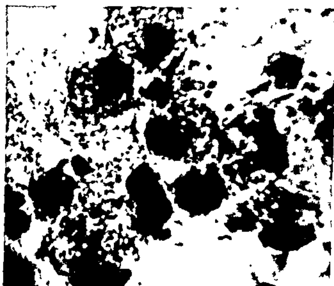


FIG. 10. TRACHEAL WASHING FROM MOUSE — IV
Four days after inoculation - ruptured cell

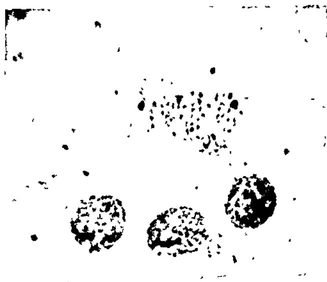
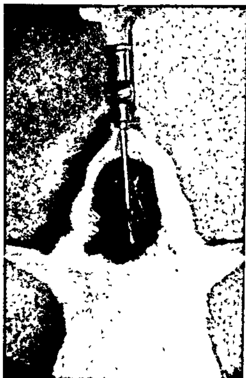


FIG. 11. TRACHEAL LAVAGE OF MOUSE



Technique of R Panthier G Cateigne and C Hannoun

FIG. 12. INOCULATION OF AMNIOTIC SAC — I
Opening of shell

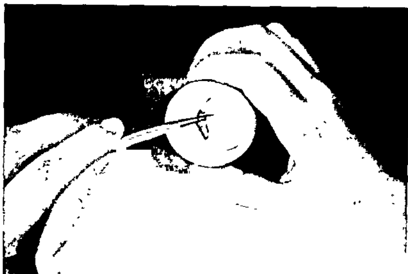


FIG 13. INOCULATION OF AMNIOTIC SAC — II
Introduction of virus

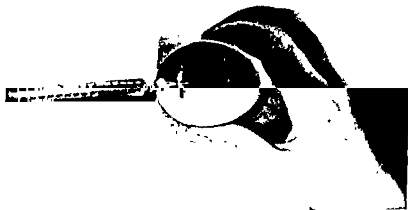


FIG 14. INOCULATION OF AMNIOTIC SAC — III
Sealing with sterile cellophane tape



FIG. 15. TRAY OF EGGS READY FOR ALLANTOIC INOCULATION

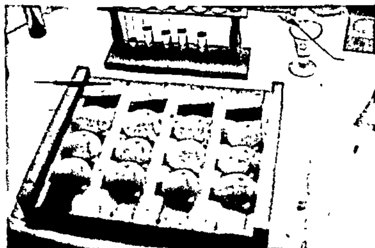


FIG. 16. INOCULATION IN ALLANTOIC CAVITY



FIG. 17. WITHDRAWAL OF ALLANTOIC FLUID — 1
Opening of air sac

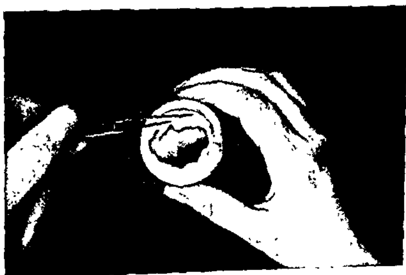


FIG. 18. WITHDRAWAL OF ALLANTOIC FLUID – II
Excision of chorio-allantoic membrane

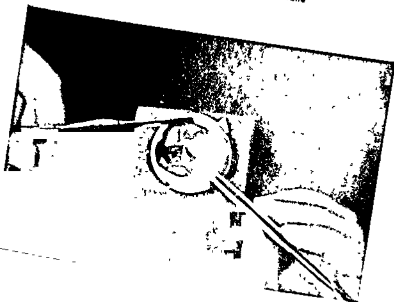
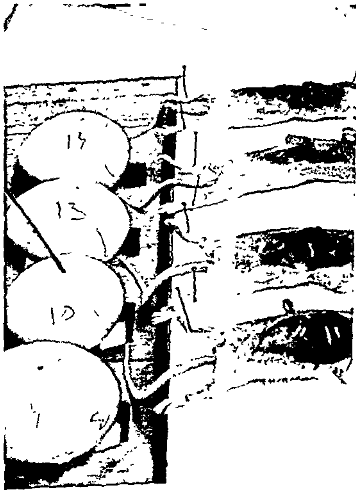


FIG. 21. INOCULATION OF EMBRYONATED EGG WITH MOUSE BLOOD — II
Immediate inoculation of egg



Technique of B Fauconniet

SELECT BIBLIOGRAPHY ON INFLUENZA

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Division of Communicable Disease Services, World Health Organization

The tremendous volume of literature on influenza prohibits a comprehensive bibliography. Earlier work has, however, been summarized repeatedly in textbooks and reviews, perhaps the most complete recent bibliography (837 references) being in van Rooyen & Rhodes *Virus diseases of man* (1948) It was for this reason that 1948 was chosen as the beginning of the period to be covered here However, since 1948 some thousand or more papers have been published, therefore further selection has been necessary This has been done, giving most space to the most recent work and successively less to older work which is covered in recent reviews and textbooks The list therefore has an uneven value, since relatively important articles published in 1948 and 1949 are omitted and articles of lesser value published in 1952 are included However, this is justified by the main purpose of this bibliography, which is to help those with limited time or facilities for studying the literature to keep themselves informed of recent work

In order to make it easier to find the literature on particular aspects of the vast subject of influenza, the bibliography has been divided into several sections by subject matter. It will be readily appreciated that frequently the subdivision is highly artificial and it must be stressed that the grouping is not an attempt to provide a bibliography of the various subjects It is intended solely to make the bibliography more convenient to use

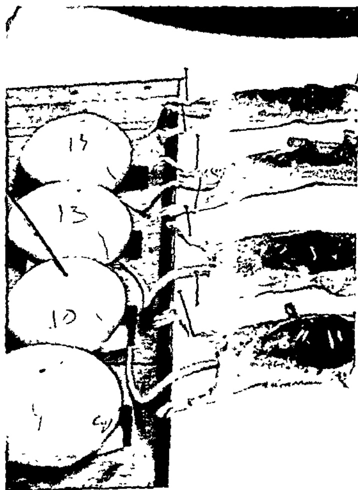
Separate lists of textbooks, monographs and general reviews are given but reviews of specific subjects have generally been included in the appropriate section

Every effort has been made to ensure accuracy but in a number of instances it has unfortunately not been possible to consult the original publication

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INDEX

The figures in bold type indicate the page numbers of articles

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